PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B10
Express Mail Label No.
EV260609015US

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 9/16, 9/127

A1

(11) International Publication Number: WO 99/04761

(43) International Publication Date: 4 February 1999 (04.02.99)

(21) International Application Number: PCT/SE98/01407

(22) International Filing Date: 22 July 1998 (22.07.98)

(30) Priority Data:
9702776–7 22 July 1997 (22.07.97) SE

(71) Applicant *(for all designated States except US)*: PHARMACIA & UPJOHN AB [SE/SE]; S–112 87 Stockholm (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ANDERSSON, Mattias [SE/SE]; Värmevägen 8, S–183 36 Täby (SE). JOHANSSON, Dorota [SE/SE]; Kilvägen 18, S–141 70 Huddinge (SE). LI, Percy [SE/SE]; Aprikosgatan 7, S–165 60 Hässelby (SE). NORRLIND, Björn [SE/SE]; Birkagatan 35, S–113 39 Stockholm (SE). WESTGREN, Bengt [SE/SE]; Blåvidegränd 34, S–162 45 Vällingby (SE).

(74) Agents: FORSLUND, Niklas et al.; Pharmacia & Upjohn AB, Patent Dept., S–112 87 Stockholm (SE).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD OF PREPARING PHARMACEUTICAL COMPOSITIONS OF LIPID PARTICLES COMPRISING A LIPID AGENT AND A PROTEIN

(57) Abstract

The invention relates to a method of preparing a composition of lipid particles comprising a bioactive protein, capable of being subjected to high shear forces without substantial loss of activity, and a lipid agent. The characterizing features of the method are the introduction of a protein preparation and a lipid agent to a homogenization station, whereupon the resulting fluid mixture of protein and lipid agent is subjected to high pressure homogenization. The so formed lipid particles are collected and if necessary further processed into a pharmaceutical formulation.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Method of preparing pharmaceutical compositions of lipid particles comprising a lipid agent and a protein.

Field of invention

The present invention relates to a method for the preparation of pharmaceutical compositions of proteins and lipids by means of high pressure homogenization, as well as pharmaceutical compositions obtainable by the process.

Background of invention

It is considered a demanding problem to define and develop a method for preparing a composition of a protein with pharmaceutical properties, suitable for large scale pharmaceutical production and resulting in a safe, efficient and clinically acceptable pharmaceutical product. One problem is to retain the stability of the protein during preparation, storage and handling. Another problem is to assure the desired pharmacokinetic and pharmacodynamic properties of the protein. It has frequently been suggested that dispersed lipid systems would constitute suitable carriers for pharmaceuticals including proteins and the mentioned problems are in many aspects the same also for such dispersed systems. It would therefore be desirable to be able to provide a method of linking proteins and lipids and thereby overcoming the mentioned problems which in many cases also relates to the dispersed lipids. In particular, it would be desirable to be able to associate the protein with a lipid carrier for improving the protein stability and the provision of a specifically designed drug delivery formulations. In certain applications, it would for example also be desirable to extend the in-vivo half life in the circulation system of the bioactive protein which otherwise risks to be enzymatically degraded before reaching the target where it should exert its beneficial activity.

A lot of efforts has been devoted in finding such suitable administration forms which maintain the bioactivity of the protein, while at the same time avoiding the involvement of carriers and formulation adjuvants which can cause clinical side-effects. Imitations of the native lipid-protein transport particles in the blood stream have been suggested as one attractive model for designing administration systems for bioactive proteins. Some important forms of these lipid particles are chylomicrons, the transporters of triglycerides which appear

in the blood stream after ingestion of lipid rich food, VLDL, LDL- and HDL-particles. These particles are mainly composed of free and esterified cholesterol, triglycerides, phospholipids and several other minor lipid components and proteins. The LDL-particles serve as transporters of cholesterol and other lipids to the cells, while the HDL-particles transport these materials to the liver for elimination. A HDL particle frequently has a disc-shaped form with an outer surface covered by a phospholipid layer and a hydrophobic core. Amphiphilic proteins, such as apolipoprotein A-I and A-II are attached to the surface by means of interaction of the hydrophobic face of their alpha helical domain with the hydrophobic part of the phospholipids

Synthetic chylomicron-like products have particularly found use as parenteral nutrients. It is a widely established technology to prepare lipid emulsions from a purified triglyceridic oil (predominantly soybean and safflower oil) and phospholipids (from egg yolk or soybeans) which are regarded as clinically acceptable for parenteral use due to their chylomicron-like emulsion droplets, generally of the size between 0.1 to 1 µm. There also exist several commercial products where such emulsions are used as carriers for lipophilic drugs which are dissolved in the dispersed lipid phase, such as Diazemuls® and Diprivan®. However, a practical complication with this type of emulsion carriers is their relative physical instability which frequently is impaired by the addition of the hydrophobic drug and lead to a break-up of the emulsion and thus making it dangerous to administer because of the risk of lipid embolism. There have been many attempts to solve this problem by adding stabilizers which, however, often are connected with undesired side-effects. The lability of such emulsions, also in connection with sterilization by high pressure steam, i.e. autoclavation and during subsequent storage, have often inhibited their use as drug parenteral drug carriers. Generally, autoclavation procedures also tend to damage many labile pharmaceuticals to be incorporated with emulsions as for example many proteins.

Liposomes have frequently been suggested as suitable vehicles for parenteral protein delivery, as for example disclosed in the article by A L Weiner in Immunomethods, 1994, Vol. 4, pp. 201-209. A liposome carrier would for example be advantageous when an improved solubilization, a sustained release (or extended half-life) or an improved targeting of the protein are desired. It is, however, acknowledged in the mentioned article that many frequently methods used to design liposomal systems often involve procedures which risk to destroy the activity of sensitive proteins, for example by denaturation and oxidation.

Moreover, in Liposome Technology, 1993 by CRC Press Inc., Vol. 1, Ch. 3, pp. 49-63: MM Brandl et al, it is disclosed how to utilize high pressure homogenization for preparing liposomes of small unilamellar quality and suitability of this technique for the reduction of vesicle size, broadness of size distribution and lamellarity of preformed multilamellar vesicle dispersions. Also the entrapment of proteins and peptides, specifically hemoglobin and insulin, is disclosed, however, the small size of the resulting vesicles is disadvantageous and the entrapment efficiencies of the proteins are low. Moreover, it is reported that the integrity and the biological function of hemoglobin is maintained, at least during short exposures to stressing conditions.

Another type of delivery system of a dispersed lipid agent which is suggested as suitable for proteins is disclosed in WO 93/06921. This system comprises colloidal lipid particles having an interior non-lamellar phase of lipids such as a reversed hexagonal phase or cubic phase which may be associated with a protein.

On the other hand, many proteins in purified form are notoriously difficult to formulate. For example, human growth hormone (hGH) exhibits poor stability in aqueous solution during storage for which reason it is advised to store preparations in a lyophilized form until its administration when it is reconstituted to an injectible solution. However, an inadvertent exposure to shear forces due to a careless reconstitution process will irrevocably lead to a loss of biological activity. For this reason especially designed means for performing a gentle reconstitution have been developed for human growth hormone as disclosed in EP 0 298 067.

There are many disclosures of synthetic HDL-particles in the literature which refer to their capacity in picking up and removing undesired lipid material in the blood stream and from the blood vessels thus making them potentially useful in therapy for treating atherosclerosis by depleting cholesterol from arterial plaques and for removing lipid soluble toxins such as endotoxins.

In Experimental Lung Res. 1984, Vol. 6, pp. 255-270: A Jonas, experimental conditions of forming complexes of the partially hydrophobic apolipoproteins and phospholipids are described in detail. It was found that, by contacting apolipoproteins with preformed phosphatidyl choline vesicles, lipid particles were spontaneously formed which could be used as analogs of HDL-particles. By mixing phosphatidyl choline and bile acids to a micellar dispersion and contacting the resultant mixture with apolipoproteins specifically

shaped, discoidal and thermodynamically stable lipid particles were formed by means of a dialysis method, subsequently called the "cholate-dialysis method".

US 4,643,988 to Research Corporation describes synthetic peptides useful in treatment of atherosclerosis with an improved amphiphatic helix and an ability to spontaneously form stable discoidal lipid particles with phospholipids which resemble native HDL-complexes. The lipid particles can be formed by contacting vesicles of phosphatidyl choline made by sonication. However, such a production method including sonication is suitable only for smaller batches of lipid particles and not for large scale pharmaceutical production.

US 5,128,318 to Rogosin Institute describes the production of reconstituted lipoprotein containing particles (HDL-particles) from plasma derived apolipoproteins which are processed to synthetic particles for parenteral administration with the addition of cholate and egg yolk phosphatidyl choline. A similar method is also disclosed in the Japanese patent application JP 61-152632 to Daiichi Seiyaku KK.

Also in WO 87/02062 to Biotechn. Res. Partners LTD, it is disclosed how to obtain a stabilized formulation by incubating a solution of recombinantly produced lipid binding protein, such as human apolipoprotein, with a conventional lipid emulsion for parenteral nutrition.

The article by G. Franceschini et al. in J. Biol. Chem., 1985, Vol. 260 (30), pp.16231-25 considers the spontaneous formation of lipid particles between apolipoprotein A-I and phosphatidyl choline. In this article, it is also revealed that Apo A-IM (Milano), the variant of apolipoprotein A-I carried by individuals shown to have a very low prevalence of atherosclerosis, has a higher affinity (association rate) to dimyristoyl phosphatidyl choline (DMPC) than regular Apo A-I. It is suggested that the mutant Apo A-IM has a slightly higher exposure of hydrophobic residues which may contribute both an accelerated catabolism and an improved tissue lipid uptake capacity of such Apo A-IM/DMPC particles.

The Canadian patent application CA 2138925 to the Swiss Red Cross discloses an improved, more industrially applicable, method of producing synthetic reconstituted high density lipoprotein (rHDL) particles from purified serum apolipoproteins and phospholipids which avoids organic solvents while resulting in less unbound, free non-complexed phospholipids (i.e. a higher yield of lipoprotein particles). Herein, it is suggested to mix an aqueous solution of apolipoproteins with an aqueous solution of phospholipid and bile acids,

whereupon the resultant mixture is incubated and protein-phospholipid particles are spontaneously formed when bile acids are removed from phospholipid/bile acid micelles with diafiltration.

The method employing the use of bile acids for making a micellar dispersion of the lipid according to the cholate-dialysis method have several drawbacks for lipid particle production, since it requires a specific separation step from the resultant mixture. Additionally bile acid residues may even in small amounts be suspected to induce side-effects after parenteral administration and may also constitute a risk of viral contamination. Moreover, the methods referred to above for preparing lipoprotein-lipid particles generally suffer from poor reproducibility and non-definable particle sizes. In particular, none of these methods are suitable in a large-scale industrial process under well-controlled conditions.

A surprisingly advantageous method is demonstrated by the present invention which meets these requirements and solve numerous problems which otherwise are associated with protein formulation, especially in large-scale production.

Description of the invention

It is an object of the present invention to provide a method for large-scale production of protein-lipid complexes which simply and economically results in lipid particle products in high yield, thus forming a composition with essentially maintained bioactivity of the protein which readily can be transformed into a formulation suitable for therapeutic utility, especially for parenteral administration, to the largest possible extent without employing any such additives which may have potential side-effects in therapy.

It is also an object of the present invention to provide a versatile method which could form a selected category of lipid particles comprising a bioactive protein, suitable for a large number of proteins and designated suitable lipid agents, in a simple manner possible to integrate with the existing or regular downstream processing of recombinant protein production.

It is another object of the present invention to provide a manufacturing process for protein-lipid complexes in the form of lipid particles which avoids subjecting the proteins to treatments leading to the loss of their bioactivity due to excessive temperatures, pH alterations which might inflict denaturation, aggregation or precipitation.

It is still another object of the present invention to provide a manufacturing process for protein-lipid complexes in the form of lipid particles after which the protein maintains its chemical identity due to oxidation of sensitive amino acids like methionine and cysteine and deamidation.

It is a further object of the present invention to provide a process for preparing lipid particles comprising a bioactive protein which has a high yield. thus avoiding remnants of large amounts of free proteins and free lipid.

It is a still further object of the present invention to provide a process for large-scale production of lipid particles comprising a bioactive protein that results in composition which readily can be transformed into a pharmacological product. for example in the form of a freeze-dried formulation.

It is a yet further object of the present invention to enable a process resulting in a pharmacologic product of lipid particles and a bioactive protein providing improved aseptic conditions without introducing sterilization methods which risks to destroy a labile protein.

Another object of the present invention is provide a process which can stabilize and modify lipid particles in a lipid dispersion by associating them with a suitable protein.

These objects of invention are attained by the inventive method directed to the preparation of a composition of lipid particles comprising a protein which is capable of being subjected to high shear forces without substantial loss of activity, and a lipid. The inventive method is generally characterized by the steps of introducing an protein preparation and a lipid agent to a homogenization station; subjecting the resultant mixture of protein and the lipid agent together to a high pressure homogenization; and finally collecting the so formed composition of lipid particles.

In addition, the present invention is also directed to a solid composition of a bioactive protein obtainable by the inventive method as well as a kit-of-parts containing such a solid composition and an aqueous reconstitution fluid.

Further details of the methods, the components forming the lipid particle and other process aids constituting different embodiments of the present invention are given below in the appended claims and in the detailed description of the invention.

Detailed description of the invention

The present invention, in its most general form, is directed to a method of preparing a composition of lipid particles comprising a protein, capable of being subjected to high shear forces without substantial loss of activity, and a lipid agent. The characterizing features of the method are the introduction of a protein preparation and a lipid agent to a homogenization station, whereupon the resulting fluid mixture of protein and lipid agent is subjected to high pressure homogenization. The so formed lipid particles are collected for an optional further processing into a pharmaceutical formulation.

The protein preparation is preferably an aqueous solution of the protein and can be obtained from downstream processing after recombinant production or any other source of protein production and may comprise varying concentrations of protein with varying purity of the desired bioactive protein. Alternatively, the protein preparation is in solid form, such as a conventional lyophilized composition. The protein preparation may simply be introduced separately from lipid into a homogenization station, for example, by separate conduits, to a running homogenization equipment.

It is to be understood that various combinations of protein preparation and lipid agent resulting in a fluid mixture are conceivable to introduce to the homogenization station in accordance with the inventive method. The protein can be introduced to the homogenization station both as an aqueous solution or as lyophilized solid preparation, whereas the lipid agent can be in the form of a an aqueous solution or be dissolved in an organic solvent. The lipid agent may also in the form of a dispersion of a lipid in aqueous solvent or be, at least partially in solid form. It is a prerequisite that any such combinations of protein preparation and lipid agent must result in homogenizable fluid and that any utilized organic solvent must be removable with efficient methods not interfering with the clinical requirements of the subsequent product.

In certain applications it is preferred that the protein preparation and the lipid agent are mixed to a homogeneous dispersion or solution before it is subjected to the high shear forces of a high pressure homogenization. It is to be understood that the premixing treatment according to the present invention can be extended to minimize the exposure of the protein to the homogenization treatment in cases when the protein is sensitive (i.e. loss of bioactivity) for extended exposure to the high shear forces during the homogenization. For the same

reason. an incubation step optionally can be introduced between the premixing and the homogenization. Alternatively. the premixing is extended to minimize extended homogenization for reasons of process economy. It is to be understood that the premixing can be performed in the same container as the homogenization is performed or in a separate station before being introduced to the homogenization station.

The lipid agent can, at least partially, be in a solid form providing a dispersion with the aqueous solution of protein. For example, a powder-formed lipid can be mixed to a homogenous dispersion before the homogenization with a conventional mixing equipment, while, on the other hand in many applications the powder formed or partially powder formed lipid agent can be introduced directly to the homogenization station.

In the present application lipid is defined as a general term for natural or synthetic compounds consisting of acyl group carriers. such as glycerol. sphingosine. cholesterol and others. to which one or more fatty acids are or could be linked. Also similar molecules that contain a substantial hydrocarbon portion may be included.

The lipid agents used in the present invention can classified into different lipid classes dependent on their polarity:

Nonpolar lipids without polar head groups. Examples of such nonpolar lipids are hydrocarbons or non-swelling amphiphiles, such as mono-, di- or triacylglycerols (glycerides), alkyl esters of fatty acids, fatty alcohols or cholesterol esters.

Polar lipids have polar head groups and exhibit surface activity, such as phospholipids and glycolipids. Dependent on their specific interactions with water they are further subdivided into the categories of swelling and soluble amphiphiles.

Amphiphatic or amphiphilic lipids are surface active and exemplified by phospholipids and glycolipids.

Polar lipids are often able to swell in the presence of water to form lipid-crystalline phases, in a structure with short range disorder and long range disorder. There are several different liquids crystalline phases. Many biological lipids such as phosphatidyl choline (PC), phosphatidyl inositol (PI) and sphingomyelin can form bilayer structures, provided that the molecules in question are roughly cylindrical in dimension. However it is also true that many major lipid components of biological systems do not form bilayer structures when isolated and placed in aqueous systems. This has been explained by the fact that the lipid molecules have a shape of a cone or an inverted cone, and thus micellar or

inverted micellar structures can be observed. Several cubic lipid-water systems of amphiphilic lipids have also been observed and there are indication of important biological functions of these lipid-water systems.

According to the present invention, the lipid agent comprises an amphiphilic lipid which is capable of forming discrete lipid particles in an aqueous medium, together with the protein, or independently of the presence of the protein, after being subjected to a high pressure homogenization.

The lipid particles generally are stabilized by the polar lipids and their morphology will vary considerably due to the nature of the protein and the lipid agent, as well as the relative amounts of these basic constituents. The present invention is suitable in producing lipid particles having a liposomal (bilayered) structure, lipid particles having the structure of an oil drop in oil-in-water emulsion, or discoidal complexes between a lipoprotein and a phospholipid, as well as other systems of discrete lipid particles stabilized in an aqueous solution, such as micelles, microemulsions, nanoparticles and dispersed hexagonal phases.

In accordance with the present invention it is preferred that the lipid agent comprises an amphiphilic agent. More preferably, the amphiphilic agent is capable of bilayer formation, e.g. a liposome membrane, in an aqueous medium and is selected among at least one of the compounds of the group of phospholipids, glycolipids and cholesterols. Suitable glycolipids are palmitoyl, stearyl or myristoyl glycosides, cholesteryl maltoside, cholesteryl glycoside, various gangliosides and the like. Examples of cholesterols are cholesterol, cholesterol acetate, dihydrocholesterol, phytosterol, sitosterol and the like.

In the present invention, the preferred amphiphilic agents are phospholipids which can be of natural origin, such as egg yolk or soybean phospholipids, or synthetic or semisynthetic origin. The phospholipids can be partially purified or fractionated to comprise pure fractions or mixtures of phosphatidyl cholines, phosphatidyl ethanolamines, phosphatidyl inositols, phosphatidic acids, phosphatidyl serines, sphingomyelin or phosphatidyl glycerols. According to specific embodiments of the present invention it is preferred to select phospholipids with defined fatty acid radicals, such as dipalmitoyl phosphatidyl choline, dioleylphosphatidyl choline, dimyristoyl phosphatidyl choline, distearoyl phosphatidyl choline, oleylpalmitoyl phosphatidyl choline and the like phosphatidyl cholines with defined acyl groups selected from naturally occurring fatty acids, generally having 8 to 22 carbon atoms. According to a specific embodiment of the present invention phosphatidyl cholines having only saturated

fatty acid residues between 14 and 18 carbon atoms are preferred, and of those dipalmitoyl phosphatidyl choline is especially preferred.

Besides the amphiphilic agent, the lipid agent may comprise, in various amounts at least one nonpolar component which can be selected among pharmaceutical acceptable oils (triglycerides) exemplified by the commonly employed vegetabilic oils such as soybean oil, safflower oil, olive oil, sesame oil, borage oil, castor oil and cottonseed oil or oils from other sources like mineral oils or marine oils including hydrogenated and/or fractionated triglycerides from such sources. Also medium chain triglycerides (MCT-oils, e.g. Miglyol®), and various synthetic or semisynthetic mono- , di- or triglycerides, such as the defined nonpolar lipids disclosed in WO 92/05571 may be used in the present invention as well as acetylated monoglycerides, or alkyl esters of fatty acids, such isopropyl myristate, ethyl oleate (see EP 0 353 267) or fatty acid alcohols, such as oleyl alcohol, cetyl alcohol or various nonpolar derivatives of cholesterol, such as cholesterol esters.

One or more complementary surface active agent can be added to the lipid agent in this invention, for example as complements to the characteristics of amphiphilic agent or to improve its lipid particle stabilizing capacity or enable an improved solubilization of the protein. Such complementary agents can be pharmaceutically acceptable non-ionic surfactants which preferably are alkylene oxide derivatives of an organic compound which contains one or more hydroxylic groups. For example ethoxylated and/or propoxylated alcohol or ester compounds or mixtures thereof are commonly available and are well known as such complements to those skilled in the art. Examples of such compounds are esters of sorbitol and fatty acids, such as sorbitan monooleate or sorbitan monopalmitate, oily sucrose esters, polyoxyethylene sorbitane fatty acid esters, polyoxyethylene sorbitol fatty acid esters, polyoxyethylene fatty acid esters, polyoxyethylene alkyl ethers, polyoxyethylene sterol ethers, polyoxyethylene-polypropoxy alkyl ethers, block polymers and cethyl ether, as well as polyoxyethylene castor oil or hydrogenated castor oil derivatives and polyglycerine fatty acid esters. Suitable non-ionic surfactants, include, but are not limited to various grades of Pluronic®, Poloxamer®, Span®, Tween®, Polysorbate®, Tyloxapol®, Emulphor® or Cremophor® and the like. The complementary surface active agents may also be of an ionic nature, such as bile duct agents, cholic acid or deoxycholic their salts and derivatives or free fatty acids, such as oleic acid, linoleic acid and others. Other ionic surface active agents are

found among cationic lipids like C10-C24: alkylamines or alkanolamine and cationic cholesterol esters.

Also other pharmacologically acceptable components can be added to the lipid agent when desired, such as antioxidants (exemplified by alpha-tocopherol) and solubilization adjuvants (exemplified by benzylalcohol).

As indicated above, the lipid agent preferably already is formulated and mixed before it is contacted with the protein solution in the premixing step or directly in the homogenization station. However, it is also conceivable within the scope of the invention to successively add one or more constituents of the lipid agent and/or protein step-wise or successively during these two processes.

According to the present invention the characteristics of the protein-lipid particles formed will vary to a large extent dependent on the composition of lipid agent and particularly on the relationship between polar and nonpolar lipids. In certain applications of the present invention, a dominating amount of polar and bilayer forming lipids may produce liposomal structures connected to protein. For example, only polar lipids in the form of phospholipids together with selected lipoproteins may form specific disc-like particles with the inventive method. If for example Apolipoprotein A1 is used, these particles have considerable stability and resemble native HDL-particle structures, so the characteristics of the protein will also considerably influence the nature of the lipid particles. On the other hand, a dominating amount of nonpolar lipids (i.e. glycerides) will form lipid particles resembling emulsion droplets which are stabilized by the polar lipids (i.e. phospholipids). Also the characteristics and the amount of the protein will influence the constitution of the lipid particle and it is obvious that dependent on the physical and chemical nature of the protein and the composition of the lipid agent different types of lipid particles will result from the inventive process. It is within the ability of the skilled person to predict the particle morphology in the resultant composition from said characteristics of the main ingredients and the remaining process parameters. The skilled person will consequently be capable of designing individual lipid agents according to the general knowledge of lipid drug delivery and by means of the inventive method form lipid particles comprising a designated protein. For these reasons the general expression “lipid particle” used herein should be given a broad meaning and be regarded to include protein complexes stabilized with lipid agent which are dispersed in an aqueous solution.

Besides the requirement to withstand the shear forces from turbulent flow and cavities resulting from the high pressure homogenization of the inventive process without substantial loss of biological activity and with substantially maintained structure, the proteins must have a degree of compatibility to the lipid agent, in order to provide stable particles comprising lipid agent and protein.

In accordance with the present invention "protein" is defined as any native occurring or recombinantly or otherwise synthetically produced bioactive protein, polypeptide or oligopeptide which is capable of a sufficient hydrophobic interaction with a lipid agent as previously defined. Sufficient hydrophobic interaction will mean that the protein at least partially interacts with the lipid agent in order to form lipid particles predominantly by hydrophobic forces rather than electrostatic attraction. In the resulting products the protein can, for example, be partially embedded into the lipid particle, penetrate into the core of lipid particle, or constitute other forms lipid protein complexes. This also excludes that the protein is simply entrapped into the aqueous phase of a liposome, as is disclosed in the aforementioned Liposome Technology, 1993 by CRC Press Inc., Vol. 1, Ch. 3, pp. 49-63: M M Brandl et al. and by A L Weiner in Immunomethods, 1994, Vol. 4, pp. 201-209. Suitable proteins preferably belong to categories 2 and 3 as defined by Y-L Lo et al. on page 805, column 2 of the article in Journ. Of Pharm. Sci, 1995, Vol. 84(7), pp. 805-814. Especially suitable proteins are membrane proteins as defined on pages 274-275 in Principles of Biochemistry, 7th Ed, E L Smith et al and lipoproteins according W V Rodrigueza et al. in Advanced Drug Delivery Reviews, 1998, Vol. 32, pp. 31-43 which frequently spontaneously interact with liposome like lipid particles to form new integrated lipid-protein particles.

According to another aspect of the present invention the protein contributes to provide the lipid particles with desired physiochemical or biological properties, such as improved stability in a dispersed system, targeting functions and functions which affect their biological distribution and elimination. In this aspect, the lipid agent may comprise a therapeutically active agent dissolved or dispersed in said lipid agent which can employ the resulting dispersed system as an improved drug delivery system. In such a case a reduction of the lipid particle size can be accomplished. At the same time the association of the protein to the surface of the lipid particle is facilitated with hydrophobic interactions as described above. This exemplified by forming a conventional lipid emulsion for parenteral use with protein

associated to the surface which can have certain parts embedded in the surface phospholipid monolayer or the oily core of the particle. It is understood that such an emulsion can comprise a specific therapeutic agent associated with the emulsion particles in manners well known to persons skilled in this art.

To be capable of hydrophobic interaction, it is preferred that the protein is at least partially lipophilic, i.e. has a lipophilic domain and/or is capable to interact with bilayer forming lipids. An example of such suitable proteins are those which exert their bioactive capacity, in connection with a surface of a biological membrane, i.e. membrane proteins. Such proteins are involved in enzymatic, transport, receptor and other functions associated with cellular membranes. Many such proteins therefore have domains which can associate with phospholipid membranes, as exemplified by so called integral proteins which are integrated directly into the bilayer of a lipid membrane. It is to be understood that functional analogs and fragments of such naturally occurring proteins can be employed with the present invention if they meet the requirements of sufficient hydrophobic interaction with the lipid agent.

More preferably, the protein has at least partially amphiphilic properties in a helix and a high capacity of interaction with bilayer forming lipids, as exemplified by the lipoproteins associated with lipid transport in the blood system. It can be expected that such proteins have a high number of exposed hydrophobic residues will have a favorable association rate in forming lipid particles with the lipid agent. Examples of especially preferred protein are such membrane proteins or lipoproteins which have a hydrophobic alpha-helix part.

It is also to be understood that proteins otherwise unsuitable to be subjected to the inventive method, due to insufficient hydrophobic interaction with lipids, readily can be adapted thereto by the introducing a group to the native protein structure which is compatible with the lipid agent. A suitable group for introducing lipid compatibility by compexing or by covalent attachment can be a peptide fragment having a number of designated amino acids which contribute to the formation of suitable characteristics, for example, an amphiphilic helix as suggested in the mentioned US 4,643,988. Also other types of groups with lipid compatible characteristics, for example having hydrophobic acyl group residues, can be associated to the native protein with covalent bonds or other types of linkages. Potentially such groups, may be complemented with targeting functions, in order to improve the direction of the drug to its active site and thus optimize its beneficial activity. The person skilled in protein chemistry will be able to find a number of such groups, suitable for designing a more

lipid agent compatible protein conjugate and to design it so that the bioactivity of the protein remains substantially unaffected. Moreover, such groups can be designed to be enzymatically cleaved in-vivo in suitable manner, without contribute to adverse effects in terms of undesired accumulation of the complementary lipid compatible substances.

Examples of proteins preferred according to specific aspects of the present invention are the apolipoproteins A-I, A-II, A-IV, B, C-I, C-II, C-III, D and E or functional analogues and derivatives thereof, such as the small peptides described in the mentioned US 4,643,988, and the like. Of these apolipoproteins, apolipoprotein A-I (ApoA-I) and its natural variants such as apolipoprotein A-IM (Milano) (Apo A-1M) can be prepared by conventional separation technology from serum or with recombinant technology, disclosed in for example WO 9312143, WO 9413819 or in WO 9807751.

According to preferred embodiment of the present invention lipoproteins (particularly apolipoproteins) having an amphiphilic helix, as defined above, will be used as the protein and the lipid agent will be substantially only phospholipids. The process will then result in disc-formed or discoidal lipid particles which resemble native HDL-particles, essentially similar to those mentioned in the articles above.

It is the particular aim of the inventive method to facilitate the hydrophobic interaction between the lipid agent and the protein while at the same time disperse lipid agent into particles. To accomplish this it is an important and characteristic feature of the present invention that the protein solution and the lipid agent, either premixed or separated, shall be introduced to a high pressure homogenization and be subjected to high pressure homogenization at conditions sufficient to form discrete lipid particles which comprise protein in a high yield, so substantially no, or only small amounts of free lipid agent and free protein remain. The high pressure homogenization serves to provide the components with a suitable amount of mechanical energy to increase their compatibility and capacity to interact. More specifically, the energy supplementation during the homogenization will facilitate the interaction of the hydrophobic parts of the lipid agent and the protein which otherwise might be shielded in an aqueous environment.

As previously mentioned, a homogenization station according to the present invention comprises a homogenizer, but may also include means for performing a premixing step of the protein solution and the lipid agent. In the premixing treatment, all the components are

manually or automatically added and co-mixed using suitable mixer. like Ystral GmbH and similar types of conventional mixers.

For the homogenization treatment. a single homogenizer can be employed and the homogenization can be carried out by one step operation, through multi-pass operation, or by a continuous operation. Also. multiple homogenizers may be employed in a series configuration. each carrying out one homogenization pass. Many commercially available homogenizers capable of being operated at a high pressure can be used in accordance with the present invention, for example Rannie high pressure homogenizer, Avestin, Gaulin homogenizers, Microfluidizers and the like.

Vessels. suitable for the homogenization preferably are conventional, commercially available vessels for pharmaceutical manufacturing, preferably jacketed vessel of stainless steel. Temperature regulation may be achieved by commercially available temperature regulators. like Julabo ATS 2 reactor temperature regulator. To provide an inert atmosphere during the manufacturing process, preferably filtered N_2 gas is used.

In order to accomplish the method of preparing the lipid particles comprising protein and successfully carry out the present invention, it is of importance that the homogenizers are operated at a high pressure which exceeds about 200 bar, but is below about 2000 bar. Preferably, the homogenizer is operated at about 600 to about 1200 bar.

In the case of a continuous operation, the homogenization time is primarily determined by the lipid particle-protein yield, the homogeneity, the particle size and zeta potential, in combination with the homogenization temperature and pressure. In the case of multi-pass operation, wherein the protein and lipid agent are subjected to several cycles of homogenization, i.e. several passages, it is rather the number of homogenization passes which is optimized instead of homogenization time. Generally, the skilled person realize that a homogenization according to the present invention requires an adaptation of pressure, process time and temperature for each individual system of protein of lipids in order to achieve desirable results in terms of yield and process efficacy, as well as maintained protein activity.

In accordance with the inventive processes, volume of the batches can be varied from small scale production in the range of 1 ml to 5 l, while up to about 20 000 l is easily achieved for normal large scale production.

In order to comply with such a potentially detrimental influence from the single high pressure homogenization, it is within the scope of the present invention to introduce plural

cycles of homogenization at gentler, somewhat lower pressure and allow for one or several intermediate rest periods in-between. The skilled person will have no difficulties to design individual running operations for specifically sensitive proteins and thereby be able to apply the inventive process for a large number of compositions to form lipid particles comprising protein.

An additional, important aspect of the present invention is the possibility to obtain improved aseptic conditions with the inventive method, since many microorganisms will not withstand high pressure homogenization. According to one embodiment of the present invention, the homogenization therefore can be performed in at least two sequences with an intermediate incubation period. The use of repeated cycles of high pressure homogenization with intermediate resting or incubation periods successively can reduce the amount of viable microorganisms in the final formulation of lipid particles, without introducing any other form of sterilization measures, such as heat or irradiation which risk to destroy the protein or adding such complementary preservative agents which may lead to problems with the tolerability of the product.

Another aspect of the present invention is to allow for an incubation step during a certain, suitable time period subsequent to the homogenization procedure, but before the collection of the resulting lipid particles for optional further processing into a pharmaceutical product. The reason being that there is tendency that the yield can increase during such a period.

As mentioned, the formulation and the process parameters must be optimized with respect to each chosen composition of protein and lipid agent. It is of high importance to consider the phase behavior, especially at different temperatures, of both the protein and the components of the lipid agent. Moreover, the capacity of the protein to withstand without being impaired in structure and/or activity of the high shear forces resulting from the high pressure homogenization must be carefully considered. Local development of heat during the treatment must also be considered, since temperatures in the homogenization process in range of 10 to 95 °C normally are encountered.

Moreover, the process parameters of the homogenization treatment, principally pressure, temperature, running time, number of homogenization cycles and incubation and the like, also will affect the lipid particle size, its size distribution and the yield of complexed lipid agent and protein. For example, it might generally be expected that the yield will

increase with more homogenization cycles, but the skilled person should be able to compromise between this advantage and other aspects resulting from the homogenization treatment.

According to the particular embodiment of the present invention when the lipid agent essentially consists of a phospholipid, it is preferred to operate at a temperature close to, or above, the phase transition temperature (Tc), at which the phospholipid is transferred from gel form into liquid crystalline form. The characteristics of the protein will also influence the efficacy of lipid particle formation, since a higher number of hydrophobic exposed residues of the protein will lead to a higher association rate with the phospholipid, whereas lower molecular weight of protein also will increase the rate of forming stable particles. Therefore, for the case when the protein is an apolipoprotein, it is predictable that the association rate is faster near the transition temperature of the phospholipid. In the case where the phospholipids are selected among phospholipids of saturated fatty acids only, it is preferred that the temperature during the homogenization is above about 42°C for dipalmitoyl phosphatidyl choline and above about 24-25°C for dimyristoyl phosphatidyl choline.

According to a first specific embodiment of the present invention the lipid agent essentially comprises phospholipids and the protein has amphiphilic properties, such as lipoproteins. An important aspect of this embodiment is to enable a protection of the amphiphilic protein by the lipid agent and provide it with improved functional characteristics including stability during preparation, purification, handling and storage and the introduction of specific biological properties, such as modulation of uptake and distribution in the body, activity, degradation rate and the like. In certain applications, it is often sufficient to employ relatively rather small amounts of lipid agent which serve to protectingly interact with local hydrophobic domains of the protein. On the other hand, certain proteins require an interaction with membrane like lipid complexes to be stabilized and/or acquire a desired bioactivity (obtain a proper orientation in lipid bilayer structures) which means that a relatively higher amount of lipid agent must be added according to the inventive methods. Preferably the phospholipids of this embodiment of the invention essentially comprises phosphatidyl cholines separated from phospholipids of native origin, such as soybean or egg yolk phospholipids or is synthetic or semi-synthetic phosphatidyl cholines with controlled content of acyl groups. Most preferred are soybean derived phosphatidyl choline, dipalmitoyl phosphatidyl choline and dimyristoyl phosphatidyl choline. The protein preferably is a human

lipoproteins, such as an apolipoprotein and the lipid particles resulting from the inventive method will find use in therapeutic or prophylactic treatment of diseases connected to lipids or lipoidal substances, including the lowering of amounts of cholesterol and endotoxins. Most preferred apolipoproteins are apolipoproteins A or E including their natural or synthetic variants, such as recombinantly produced mutant apolipoprotein A-I_{Milano}. The phospholipids preferably are added to an aqueous protein solution in a premixing step in weight relationship of lipid to protein of from about 1:100 to about 10:1 (w/w). As a reference to the lower limit of lipid to protein ratio, it is referred to albumin in its native fatty acid transporting function where the lipid to protein ratio is about 1:100 (w/w). Preferably, the amount of lipid agent to protein in accordance with this embodiment of the present invention is from about 1:4 to about 4:1 and more preferably between about 1:1 to about 3:1. Generally, it is aimed to obtain a yield of lipid-protein complex over 90 % and preferably close to 100 %, so little or almost no non-associated protein or lipid agent is obtained in the resulting lipid particle composition. It is also highly desirable to have as low amounts of phospholipids as possible in any injectible preparation, since excessive amounts may produce bilayered vesicles (e.g. liposomes) which conceivably might induce side effects in the subject receiving such a preparation. The high pressure homogenization is performed during a suitable time and temperature at a suitable pressure in the range from about 200 bar to about 1500 bar, preferably from about 600 to about 1200 bar and. The homogenization can be performed in one or several periods with an intermediate rest period of a suitable duration with an optional subsequent incubation step. This method results in a high yield from about 90 to 100% of discoidal lipid particles comprised of phospholipids and protein with a particle size ranging from about 7 to about 25 nm. The protein incorporated in the lipid particles formed by the inventive method has maintained its chemical identity in terms of oxidation and deamidation. The lipid particles therefore will be capable of exerting the same biological activity as protein not subjected to homogenization when incorporated in a final pharmaceutical preparation.

According to a second specific embodiment of the invention, the solution of an amphiphilic protein is contacted with an aqueous lipid dispersion, preferably an oil-in-water emulsion (lipid emulsion) in a method including high pressure homogenization . The lipid dispersion preferably is a conventional emulsion for parenteral use which have clinical acceptance, such as Intralipid, Liposyn or other emulsions based on a triglyceride oil of vegetable origin (soybean, safflower oils) and a clinically acceptable emulsifier, such as egg

yolk or soybean phospholipids. The skilled person will be able to vary the contents and composition of the emulsion, for example in accordance with discussion of suitable nonpolar lipids above. It is preferred that the emulsion comprises about 1 to 50 % (w/w) of an oil phase and about 0.05 to 30 % (w/w) of a phospholipid emulsifier and that the oil phase comprises triglyceride oils (triglycerides preferably of long chain saturated or unsaturated fatty acid and/or medium chain fatty acids) or alkyl esters of fatty acids suitable for parenteral administration. Person skilled in emulsion technology will readily find suitable lipid emulsions which are applicable in the inventive method. The relationship between lipid agent (nonpolar lipid and emulsifier) and protein typically may vary between about 500:1 to about 10:1 (w/w) and preferably between about 60:1 and 20:1. However, the skilled person will be able deviate from these recommendations in certain applications required by the characteristics of the lipid agent and the protein and the specific clinical utility of the product. For example, it can be desirable to retain a high nutritional value of the lipid particle composition or to comprise a high amount of a lipid in order to be able to incorporate a lipid soluble additional therapeutic agent in the lipid particles. Alternatively the lipids have a therapeutic and diagnostic value *per se*, for example as carriers of beneficial fatty acids or diagnostic value or as contrast agents having for example iodinated fatty acids for delivery to a target organ. In such applications, the inventive method is useful for linking proteins to the lipids and thereby modify the distribution and elimination of the resulting lipid particles. The inventive method can according to this aspect be used to obtain a protein coating of the lipid particles which contains a relatively low amount of protein compared to lipid. Such a coating of the lipid particle can result in a modified interaction with the natural recognition of the immune system as accomplished by linking antibodies to the lipids with the inventive method. In such a case, the ratio lipid to protein can be extremely high, since it is conceivable that as few as less than thousand protein molecules are sufficient to associate to the outer region of the lipid particle and yet obtain significant results. On the other hand, the proteins can also be employed to change the chemical physical characteristics of the dispersed lipid particles by using the inventive method. For this case a higher protein load can be required resulting a smaller lipid to protein ratio. The high pressure homogenization of lipid emulsion and protein is performed during a suitable time and at suitable temperature at a high pressure preferably not exceeding about 2000 bar, more preferably being in the range from about 200 bar to about 1500 bar and most preferably from about 600 to about 1200 bar. The protein is preferably is

an apolipoprotein which will find use in therapeutic or prophylactic treatment of diseases connected to lipids or lipoidal substances, including lowering of amounts of cholesterol and endotoxins. Most preferred apolipoproteins consists apolipoproteins A or E including their natural or synthetic variants, such as recombinantly produced mutant apolipoprotein A-I$_{\text{Milano}}$. The lipid particles resulting from the homogenization with an emulsion preferably have a mean particle size less than about 1 μm and preferably in the range of about 0.1 to 0.5 μm. The lipid particles with associated protein will to different extents have a modified zeta potential in comparison to the lipid particles of the original emulsion which may provide the resulting preparation with an improved physical stability which also is an indication that the amphiphilic protein (at least partially) is associated with the surface layer of the lipid particles. Generally, a non-favorable zeta potential should be avoided due to risks of aggregation of lipid particles which might inflict embolism after intravenous administration. It is therefore often necessary to induce a contribution to the net charge of the resulting lipid particles. This can be accomplished by conventional measures, such as a pH change, introduction of a supplementary stabilizing charged agent and the like.

After the homogenization step of the inventive methods is completed referred to above is completed, the lipid particles of each batch are collected with conventional measures and equipment, which may include centrifugation or filtration to improve the concentration and the purification of the lipid particle product, as well as conventional processing to obtain an aseptic product.

The so formed product can the be subjected to conventional lyophilization optionally with the addition of suitable excipients, so as to form a final pharmaceutical solid product, suitable for long-term storage and subsequent reconstitution with an aqueous fluid, just prior to its parenteral administration, for example by intravenous injection. Reconstitution may be accomplished by addition of buffer solution containing suitable excipients with respect to tonicity as well as rate of dissolution. Suitable buffer includes sodium phosphate, histidine and the like. Excipients include polyols, like mannitol, glycerol, saccharose and amino acids.

According to an embodiment of the present invention the lyophilization, can be performed batch-wise, *in-situ,* in designated chambers of conventional multi-chamber cartridges, or alternatively directly in a chamber located in the barrel of a multi-chamber injection device. These devices will form kit-of-parts comprising the one or plural dosages of solid composition in a chamber separated from a neighboring chamber, storing an aqueous

parenterally administerable reconstitution fluid, by means of a movable wall which can be displaced in order to form an injectible fluid, just before the desired administration. The skilled person can readily find several examples of such syringes or cartridges which can be operated by pen type syringe devices (see e.g. EP 298 067).

Examplifying description of the invention

Fig. 1 demonstrates peptide maps of the mutant apolipoprotein A-I_{Milano}, before homogenization together with phospholipids.

Fig. 2 demonstrates a peptide map of the mutant apolipoprotein A-I_{Milano} after high pressure homogenization with phospholipids.

Fig. 3 demonstrates a reverse phase chromatogram (HyTach) of the reduced mutant apolipoprotein A-I_{Milano}, before homogenization together with phospholipids.

Fig. 4 demonstrates a reverse phase chromatogram (HyTach) of the reduced mutant apolipoprotein A-I_{Milano} after high pressure homogenization with DPPC.

Fig. 5 shows an IEF-diagram comparing apolipoprotein A-I_{Milano} treated with lipid and homogenized according the present invention in comparison to the same, untreated protein.

Fig. 6 shows zeta potential of a 20 % soybean emulsion comprising apolipoprotein A-I_{milano} when compared to references.

Fig. 7 shows zeta particle sizes of a incubated mixture comprising a 20 % soybean emulsion and apolipoprotein A-$I_{Milano.}$ when compared to references.

<u>Example 1</u>

0.687 g SPC (soy bean phosphatidyl choline) is mixed in a premixing step in a jacketed vessel with 45.80 g of a protein solution comprising apolipoprotein A-I_{Milano},(Apo A-IM) obtained from the downstream process from recombinant production with a protein

concentration of 12 mg/ml, in sodium phosphate buffer. The ratio lipid to protein was thus 1.25 : 1. The temperature was regulated to 60 °C by a Julabo ATS 2 reactor temperature regulator. An Ystral mixer in a rotor and stator configuration is used as mixer with stirring speed controlled at 2000 rpm. After 10 minutes of pre-mixing, the mixture is homogenized in a mini Rannie homogenizer, Mini-Lab type 7.30 VH, at a pressure of 1000 bar, 60 °C continuously for 5 minutes. This method results in the formation of transparent solution of lipid-protein particles with a structure resembling disc-formed native HDL-like complex. The yield of protein incorporated into lipid complexes as detected by native-PAGE is 98% (according to the method described below) In a replicate experiment the yield was 100 %, demonstrating the reproducibility of the process.

Example 2

0.47 g of recombinant apolipoprotein A-I_{Milano},(Apo A-IM), (9.6 mg protein/ml in a phosphate buffer) and soybean phosphatidyl choline (SPC) added to 1:1 lipid/protein ratio (w/w) was premixed at 60 °C for 10 minutes and then homogenized for 5 minutes at 60 °C and 1000 bar in the mini-Rannie homogenizer of Example 1. All protein was incorporated into 8 nm lipid-protein particles as measured by n-PAGE (according to the method described below) . Accordingly, it is demonstrated that a high incorporation of apolipoprotein A-I_{Milano} into lipid-protein particles is obtained in the homogenization process although a low ratio of lipid to protein is used.

A similar experiment was performed with same components and during the same conditions as above, except that the homogenization was performed at 560 bars for 7 minutes at 60 °C. According to subsequently performed chromatographic and electrophoretic analyses, the protein is essentially unchanged in the process. This is exemplified in Fig. 1 and Fig. 2, revealing data from peptide mapping (according to the method described below) of the apolipoprotein A-I_{Milano} and the same protein after homogenization with lipid to form protein-lipid complexes.

Example 3

69.00 g of a solution of recombinantly produced Apo A-IM (19.8 mg/ml solution in water) was homogenized together with 4.485g dipalmitoyl phosphatidyl choline (DPPC) at 42 °C and 1000 bar for 60 minutes with a Mini Rannie homogenizer. Before homogenization, the mixture was pre-mixed for 5 minutes at 42 °C at 2000 rpm in a nitrogen atmosphere. The ratio DPPC : Apo A-IM was 3.3 : 1 (w/w). Analysis for protein denaturation (deamidation, oxidation or aggregation) was made by peptide mapping, isoelectric focusing, size exclusion chromatography and reverse phase chromatography (HyTach). The data for the Apo A-IM protein in the processed material was in compliance with protein material which was not subjected to homogenization. as demonstrated by HyTach data in Fig. 3 and Fig. 4 (measured with a method disclosed below). The amount of protein incorporated into lipoprotein particles (in the size range 7.7-15.7 nm) was 100 % according to densitometric scans of n-PAGE stained gels (see below). The corresponding hydrodynamic radius of the reference protein was estimated to 7.3 nm. To confirm that the particles contained phospholipid as well, the n-PAGE gels were also stained for lipids. This example demonstrates the efficient formation of lipid-protein complexes at a different composition and process condition , and the fact that the protein is essentially unaffected by the mechanical treatment together with lipid.

Example 4

75 g of a solution of recombinantly produced Apo A-IM, 15 mg/ml with dipalmitoyl phosphatidyl choline (DPPC) added at a ratio of 2.9:1 (w/w) in 10 mM sodium phosphate (pH=7.5) was homogenized after a 5 minute pre-mixing step under the same conditions as in Example 3. The homogenization was performed with a first homogenization period of 7.5 minutes at 60 °C and 1000 bar followed by a second homogenizing period of 5 minutes at 40 °C and 1000 bar. After the first homogenizing period the amount of protein incorporated in lipid-protein particles of 7.7 to 25 nm was 78 % which increased to 94 % after the second homogenizing period.

There were no aggregation, truncation, deamidation or oxidation observed by chromatographic or electrophoretic methods in the proteins subjected to the two-step homogenization process.

Example 5

263 g of dipalmitoyl phosphatidyl choline (DPPC) was added to 6.7 kg of a solution of recombinantly produced Apo A-IM, 13 mg/ml, in 10 mM sodium phosphate (pH 7.5). The material was pre-mixed for 10 minutes at 50 °C with an Ystral X 20 D-mix equipment. The material was thereafter homogenized at 42 °C and 900 bar in a high pressure homogenization of the type Lab. Rannie 12.51-H. The homogenization was performed for 35 passages where each passage has duration of 3 minutes. After the homogenization 243 g sackaros and 31 g mannitol were added and dissolved, whereupon the solution was sterile filtered, aseptically filled and freeze-dried. Subsequent electrophoretic and chromatographic analyzes of the product after its reconstitution showed that the protein was in compliance with the protein material not subjected to homogenization. This is demonstrated with the IEF data (Fig. 5) of a sample of the homogenized, final protein preparation according to this example in comparison to an untreated protein (according to a method disclosed below). The efficacy of the process is shown by n-PAGE tests where 99 % of the protein was incorporated into lipoprotein particles in the size range 7.7 to 25 nm. This example demonstrates that high quality protein-lipid complexes can be produced in an efficient process with a comparatively low lipid/protein ratio in a scaled up process.

Example 6

0.72 g of dimyristoyl phosphatidyl choline (DMPC) was added to 48.10 g solution of recombinantly produced Apo A-IM, in an Ystral mixer, giving a lipid : protein ratio of 1:1. Pre-mixing was performed for 3 minutes at 60 °C and 2000 rpm under nitrogen atmosphere before a homogenization for 7 minutes at the same temperature at 560 bar in a Rannie homogenizer. The so prepared lipid-protein particles were cooled to less than 30 °C. The equivalent hydrodynamic radius of the discoidal complexes was determined to 15.1 nm (z average), by Malvern 4700 dynamic light scattering equipment.

Analysis of the protein-lipid complex according to the invention by reverse phase chromatography of the reduced protein (HyTach analysis) verifies the maintained integrity of the protein in the described process. Thus the level of oxidized protein is below

the level of quantification for the method (0-3 %), whereas the total level of modified protein is 12 % compared to 8 % in a reference sample of the protein.

Example 7

This example comprises the preparation of an emulsion formulation of soybean oil in water, with the protein r-ApoA-1M using a high shear device. The formulation is compared to a reference emulsion made without added protein and also with an emulsion incubated with the same protein.

Preparation

A test emulsion (20 % soybean oil and 1.2 % egg phospholipids) containing r-ApoA-IM and a corresponding reference emulsion without protein was prepared in two steps. First a concentrated coarse emulsion was made by dispersing 60 g of soybean oil and 3.6 g of purified egg phospholipids in 113 g of distilled water, followed by coarse homogenization in a Rannie homogenizer (type 7.30 VH) at 60 °C, with a small addition of a 1 N NaOH solution. The coarse emulsion was divided into two parts, one of which was kept at 60 °C. To the other part (92.7 g) was added 62.1 g of distilled water and the emulsion was homogenized at 800 bars of pressure in the same Rannie homogenizer for 6 minutes at 60°. The resulting reference emulsion (A), was cooled to room temperature and dispensed on 20 ml vials.

The first part of the coarse emulsion above, was reintroduced to the homogenizer, and 62.2 g of a solution of r-ApoA-IM in distilled water (22.5 mg per ml) was added. The emulsion was homogenized at 800 bars of pressure in the Rannie homogenizer for 6 minutes at 60°. The resulting test emulsion (B), was cooled to room temperature and dispensed on 20 ml vials.

In a similar incubation experiment, an incubation sample (preparation C) of Intralipid 20 % and r-ApoA-1M was prepared by gentle mixing of 8 g of Intralipid 20 % with 2 g of the r-ApoA-1M solution above (containing 22.5 mg of r-ApoA-1M per ml), at room temperature. A reference preparation (D) was prepared in a similar way, using distilled water instead of the protein solution.

Evaluation

The preparations A, B and C were immediately evaluated with respect to mean particle size and particle charge, using a MALVERN Zeta Sizer 4, with the samples diluted in a 2 mM TAPS buffer pH 8.4 . The B preparation was also evaluated after 60 hours storage of the preparations at 55° C (B, incubated). The C and D preparations were stored at 25° C for 20 hours and at 55° C for an additional 60 hours. Separate vials of preparation A and B were evaluated for shake stability by shaking for 66 hours at room temperature.

Results

The results are demonstrated in Fig. 6 (particle sizes) and Fig. 7 (particle surface charge) for the emulsions in Preparations A. B , before and after incubation (B).

For preparation C and D, there is no change in particle size during the incubation period. During the incubation period the particle charge in preparation C increases by 8 mV compared to 4 mV for the reference (D). There is thus a clear indication of absorption of r-ApoA-1M to the emulsion particles during incubation. However, the absorption process is very slow and requires elevated temperatures.

Preparation B, shows a remarkably reduction in particle size (180.9 nm) compared to A, the reference preparation (247.5 nm), and the size does not change during the subsequent incubation step. Similarly, there is a large difference in emulsion particle charge at homogenization (48.5 mV for test preparation B compared to 38.1 mV for preparation A), and the charge continues to increase to 61.7 mV during the subsequent incubation step.

These data indicate that recombinantly produced ApoA-IM adsorbs to, and interact with the emulsion particles to a high extent during homogenization . This influences not only the charge of the emulsion droplets but the protein also acts a an emulsifier, which enables the emulsion droplets to assume a higher surface curvature, which leads to a smaller mean particle size. During the subsequent incubation at 55°C, the continued increase in particle charge indicates a continued adsorption of protein to the surface of the emulsion particles. In the experiment where the protein is incubated with a similar emulsion, the interaction is quite different, in the sense that the particle size is essentially unchanged during the extended incubation period, and the particle charge changes to a much smaller extent during incubation.

A further indication of a high adsorption of protein to the emulsion droplets in preparation B is given by the shaking test of the preparation A and B. After the shaking period (66 hours) the preparation A demonstrated a large oil droplet on the surface of the emulsion, and also large quantities of oil on the glass surface, whereas preparation B had essentially no visible oil. This difference is explained by the stabilization of the protein-containing emulsion due to the higher charge (as indicated above) on the emulsion droplets.

These experiments demonstrate that efficient association of a partially hydrophobic protein to emulsion particles can be accomplished when the interaction between the components is facilitated by the use of a high shear device and also followed by an incubation period.

Analytical evaluation procedures

The size distribution and relative amount of the formulated recombinant lipoprotein A-IM/phospholipid were estimated by densitometric evaluation after separation by nondenaturating polyacrylamide gradient gel electrophoresis (n-PAGE) on Novex gels with a 4-20 % linear gradient of acrylamide. The separation is based on size and the size distribution is estimated by comparing the samples with globular proteins with known Stoke's diameters, run on each gel. After electrophoresis the protein moieties are visualized by staining with Coomassie Brilliant Blue. The stained gels are scanned on a densitometer whereupon the gel images are processed and evaluated by the ImageMaster software. Apparent sizes and relative amounts of the protein stained bands are calculated.

Peptide mapping for identity testing of apolipoprotein A-IM was performed with a fragmentation with endoproteinase Lys-C digestion enzyme and analysis by reversed phase HPLC using a 2.1 mm i.d. Zorbax SB-C8 column. The peptide fragments are separated and detection is performed with UV detection at 220 nm. The sample peptide is compared with standard material digest. Oxidized forms, truncated forms and unknown new peaks are observed with this method. This separation technique is based on reversed phase chromatography at pH 2 with a step gradient from 3 % acetonitrile to about 38 %. A Zorbax Staplebound columns with 2.1 mm inner diameter was used with flow rate of 0.21 mm during a 90 minute analysis.

Quantitative determination and purity of recombinant apolipoprotein A-IM was performed with reversed phase HPLC utilizing a HyTach column. This method aims to differentiate between i) the monomer form of recombinant apolipoprotein A-IM (r-ApoA-IM) and modified monomeric forms of the protein and ii) the intact dimer form and modified dimeric forms of the protein. The differentiation between intact and modified forms of the protein is made possible by first reducing the protein with mercaptoethanol to ensure that dimeric forms not are present. The impurity content of the r-ApoA-IM samples are expressed as the area percentage of changed monomeric forms including unknown peaks of the total peak area seen in the gradient interval. To differentiate between intact r-ApoA-IM dimer and dimeric variants, the reduction procedure is omitted. The dimeric forms are separated from the monomeric ones, thus all of the present r-ApoA-IM forms are determined in the analysis. The separation technique, reversed phase chromatography, mainly separates according to hydrophobic differences of the molecule. This is useful for the separation of truncated forms and degraded forms from intact protein, due to differences in hydrophobicity. Quantitation of the intact r-ApoA-IM monomer (after reduction of the protein) is determined on the peak corresponding to intact monomer only. The concentration is determined by constructing a calibration graph with r-ApoA-IM reference material at four levels. The separation is performed with 2 µm non-porous C18 modified silica particles. The mobile phase consists of 0.25 % trifluoroacetic acid in water-isopropanol mixture. The protein is eluated in a gradient run with increasing organic solvent from 52 to 62%.

The IEF analysis of r-ApoA-IM was performed on the gel Immobiline DryPlate 4-7, a polyacrylamide gel with an immobilized linear pH gradient. The proteins are electrophoretically separated in the pH gradient according to their isoelectric points (pI), i.e. when the net charge within the molecule is zero.

Claims

1. A method of preparing a composition of lipid particles comprising a lipid agent and a protein **characterized by**:

(i) introducing a protein preparation and a lipid agent to a homogenization station;

(ii) subjecting resulting fluid mixture of protein and lipid agent to high pressure homogenization; and

(iii) collecting the so formed composition of lipid particles.

2. A method according to claim 1 **characterized by** mixing the protein preparation and the lipid agent to a homogenous fluid mixture before the homogenization.

3. A method according to claims 1 or 2, wherein the protein preparation is an aqueous solution of the protein.

4. A method according to any preceding claim, wherein the high pressure homogenization is performed at a pressure of at least about 200 bar, but not exceeding about 2000 bar.

5. A method according to claim 4, wherein the high pressure homogenization is performed at pressure of about 600 to about 1200 bar.

6. A method according to any of claims 1 to 5, wherein the homogenization is performed in at least two sequences with an intermediate incubation period.

7. A method according to any of claims 1 to 6 allowing for an incubation period after the homogenization, but before collecting the lipid particles.

8. A method according to any of claims 1 to 7, wherein the lipid agent, at least partially, can be in solid form providing a dispersion with the protein preparation before the homogenization.

9. A method according to any of claims 1 to 8, wherein the lipid agent comprises amphiphilic compounds.

10. A method according to claim 9, wherein the lipid agent essentially comprises phospholipids.

11. A method according to claim 10, wherein the phospholipids consist of phosphatidyl choline of natural or synthetic origin with a defined fatty acid composition.

12. A method according to claim 11, wherein the phosphatidyl choline is selected among egg yolk or soybean phosphatidyl cholines or a phosphatidyl choline having acyl groups of fatty acids with between 14 or 18 carbon atoms.

13. A method according to claim 12, wherein the phosphatidyl choline is dipalmitoyl phosphatidyl choline.

14. A method according to any of claims 9 to 13, wherein the lipid agent besides amphiphilic compounds further comprises at least one nonpolar lipid.

15. A method according to claim 14 wherein the nonpolar lipid is selected among glycerylesters, alkylesters and cholesterol including its nonpolar derivatives.

16. A method according to any preceding claim, wherein the amount of lipid agent in relation to protein is about 1:100 to about 10:1 (w/w).

17. A method according to claim 16, wherein the amount of lipid agent in relation protein is about 1:4 to about 4:1.

18. a method according to any of claims 1 to 7 characterized in that the lipid agent is a dispersion of lipids in an aqueous medium.

19. A method according to claim 18 **characterized in that** the dispersion of lipids is an oil-in-water emulsion.

20. A method according to claim 19 **characterized in that** the emulsion comprises about 1 to 50 % (w/w) of an oil phase and about 0.5 to 10 % (w/w) of a phospholipid emulsifier.

21. A method according to claim 20 **characterized in that** the oil phase comprises triglyceride oils or alkyl esters of fatty acids suitable for parenteral administration.

22. A method according to any of claims 19 to 21 resulting in lipid particles having a mean particle size less than about 1 μm.

23. A method according to claim 22, wherein the obtained lipid particles have a mean particle size range of about 0.1 to 0.5 μm.

24. A method according to any of claims 18 to 24 wherein the amount of lipid agent in relation to protein is about 500:1 to about 10:1 (w/w).

25. A method according to any of claims 18 to 24 wherein a therapeutically active agent is dissolved or dispersed in the lipid particles.

26. A method according to any preceding claim wherein an additional agent selected from a group consisting of polyols, mono-, di- and polysaccharides and amino acids is added to the mixture of protein and lipid.

27. A method according to claim 1, wherein the protein is capable of hydrophobic interaction with the lipid agent.

28. A method according to claim 27, wherein the protein is at least partially lipophilic.

29. A method according to claim 28 wherein the protein is selected among membrane proteins and lipoproteins or active fragments thereof.

30. A method according to claim 27, wherein the protein has an alpha-helix domain capable of hydrophobic interaction with the lipid agent.

31. A method according to any of claims 1 to 30, wherein the protein is bioactive and capable of being subjected to high shear force without substantial loss of its biological activity.

32. A method according to claims 1 to 7 or 18 to 31, wherein the protein exerts a stabilizing influence on the resulting lipid particles

33. A method according to claim 1, wherein said protein is an apolipoprotein.

34. A method according to claim 33, wherein said protein is selected from a group of apolipoproteins consisting of apolipoprotein A or E including their natural or synthetic variants.

35. A method of preparing a composition of lipid particles containing an least partially lipophilic protein, capable of being subjected to mechanical energy without substantial loss according to any previous claim **characterized by** purifying and concentrating the composition of lipid particles to a pharmaceutically acceptable composition.

36. A method according to any preceding claim **characterized by** lyophilizing the so formed composition to a final pharmaceutical product.

37. A composition of lipid and a bioactive protein as obtained by the method according to any of claims 1 to 36 **characterized in that** the protein essentially has maintained its chemical identity without being subjected to oxidization or deamidation.

38. A multi-chamber container comprising the lyophilized composition according to claim 36 separately stored from an aqueous fluid for its reconstitution to a parenterally administerable fluid.

39. A container according to claim 38 having said lipid and said fluid stored in different chambers separated by at least one movable wall which can be displaced in order to form an injectible fluid just prior to a parenteral administration.

40. A container to claim 39 **characterized in** that provided with means for being actuated by an injection device.

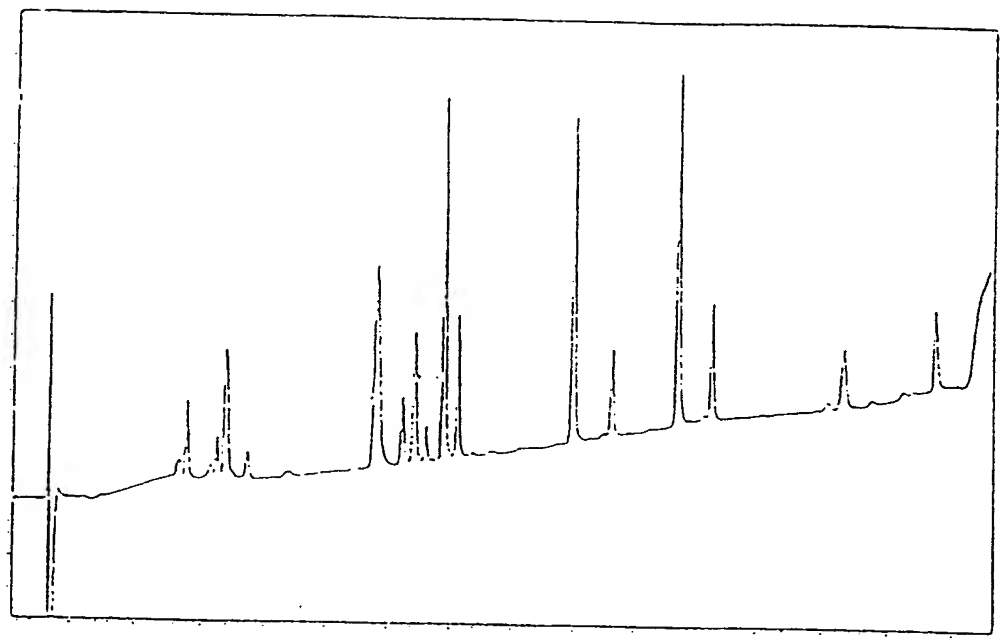


Fig. 1

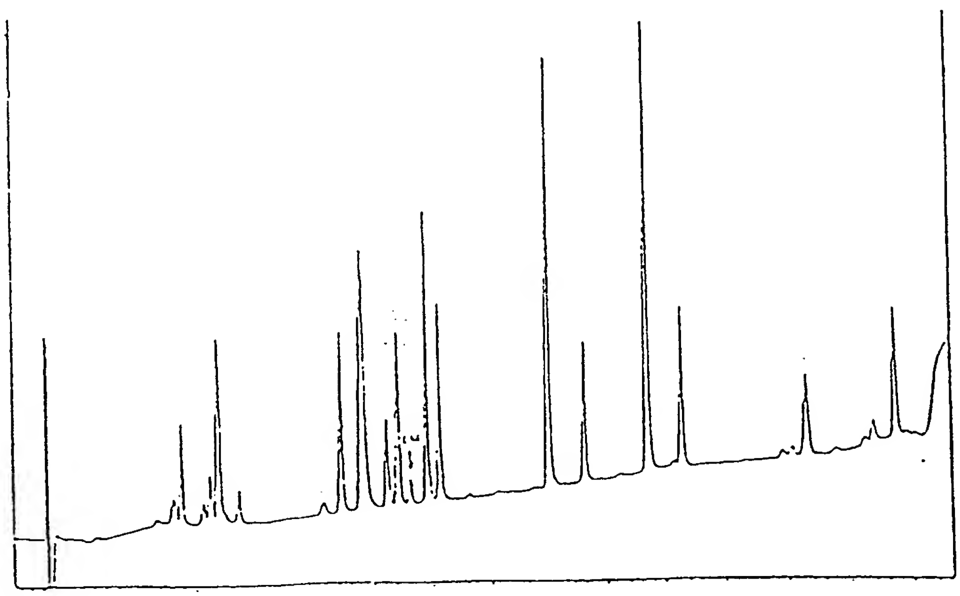


Fig. 2

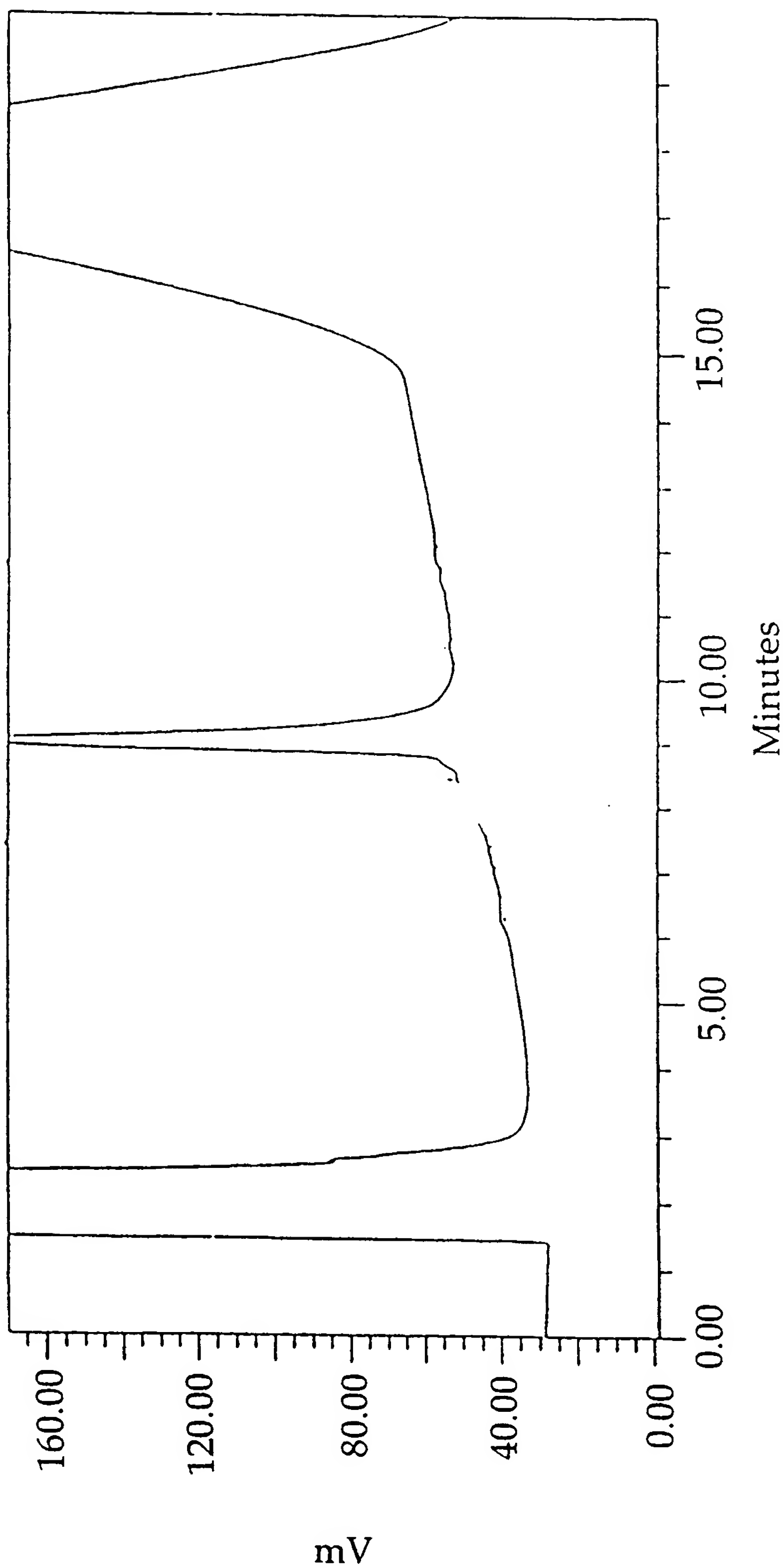


Fig. 3

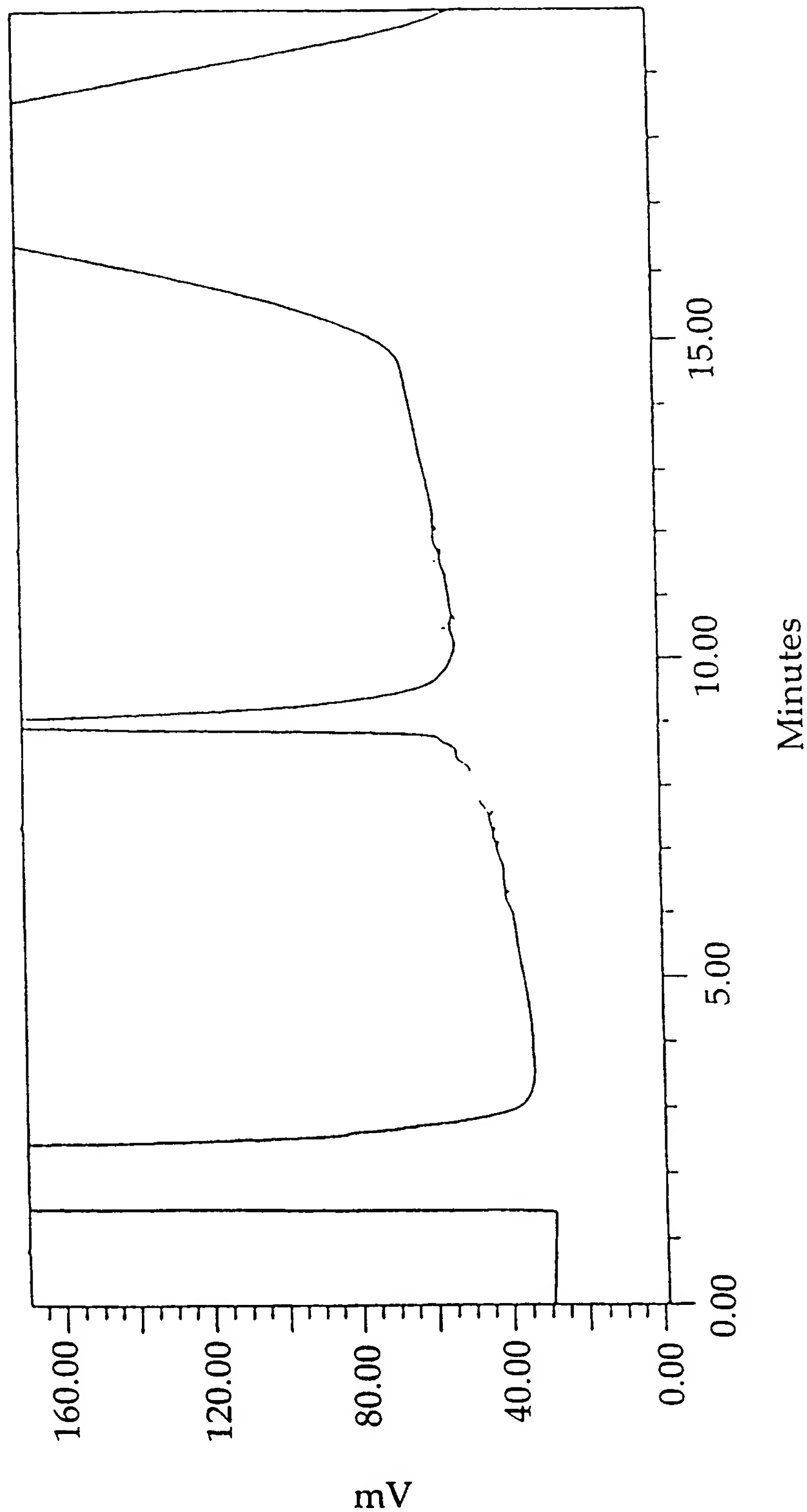


Fig. 4

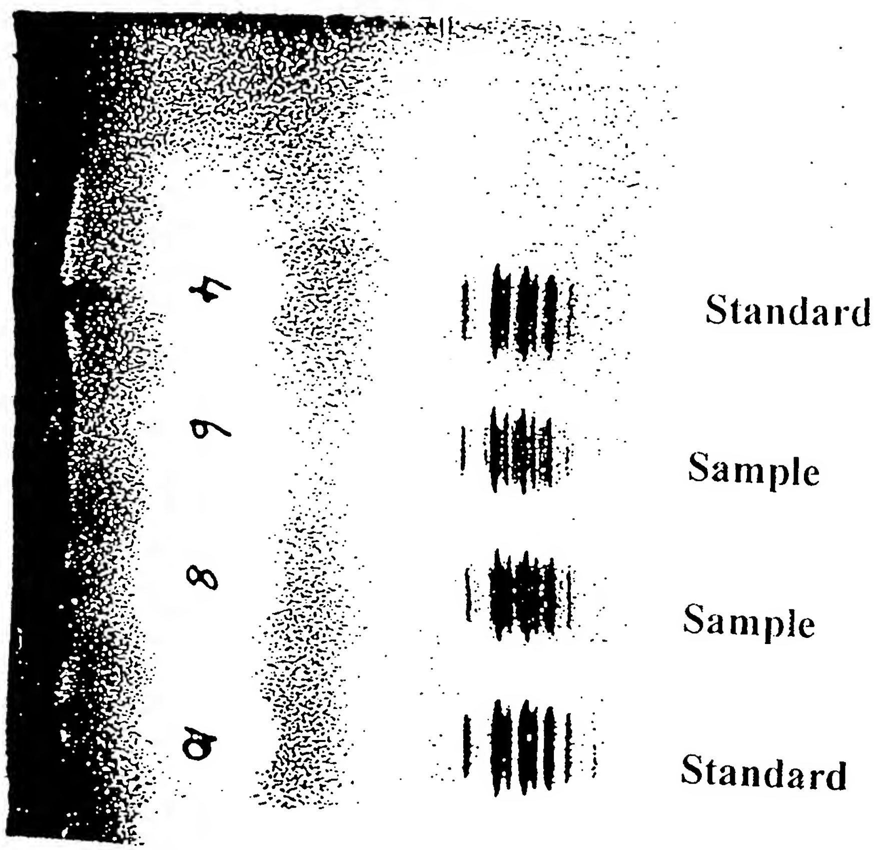
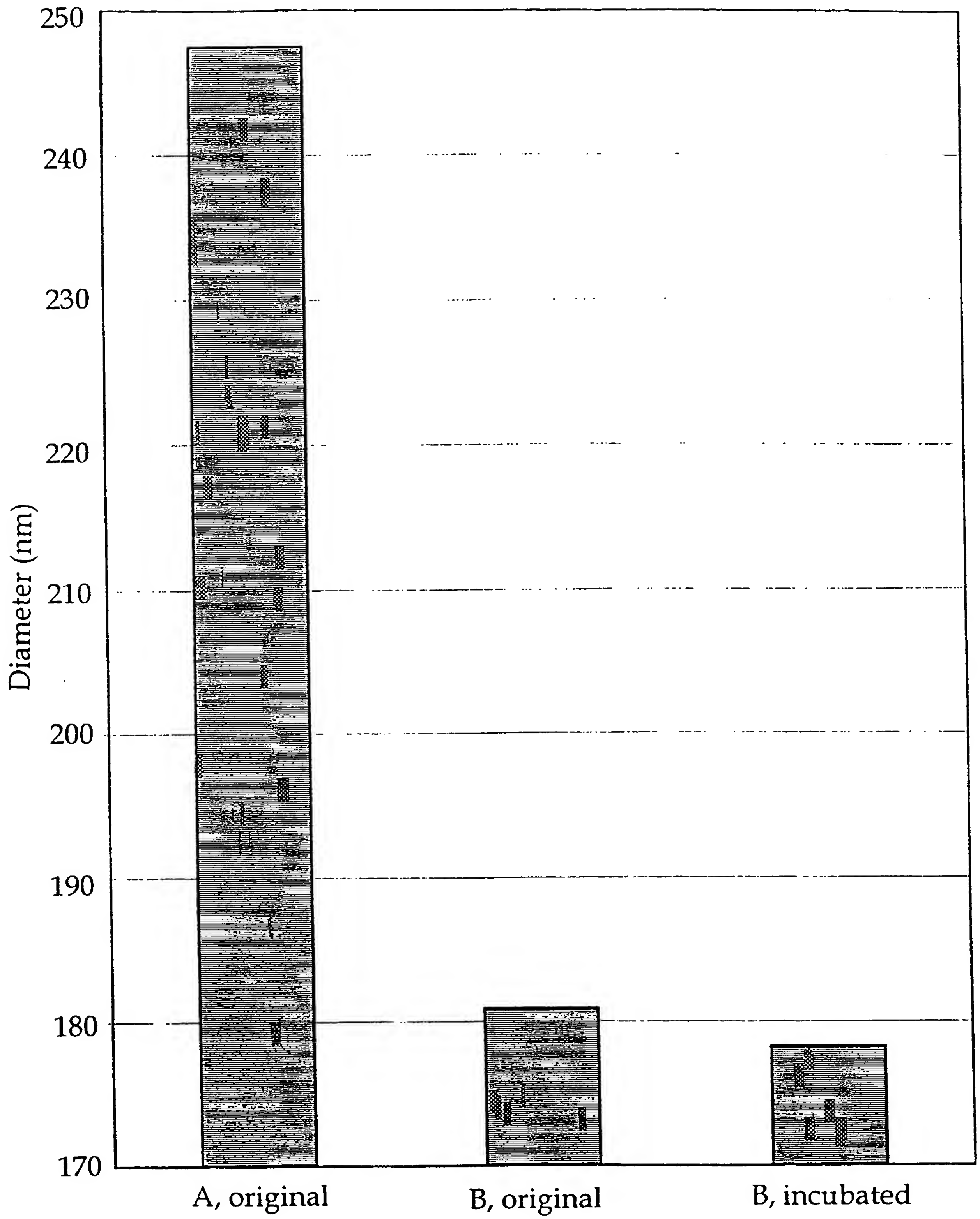


Fig. 5



250
240
230
220
210
200
190
180
170
Diameter (nm)
A, original
B, original
B, incubated

Fig. 6

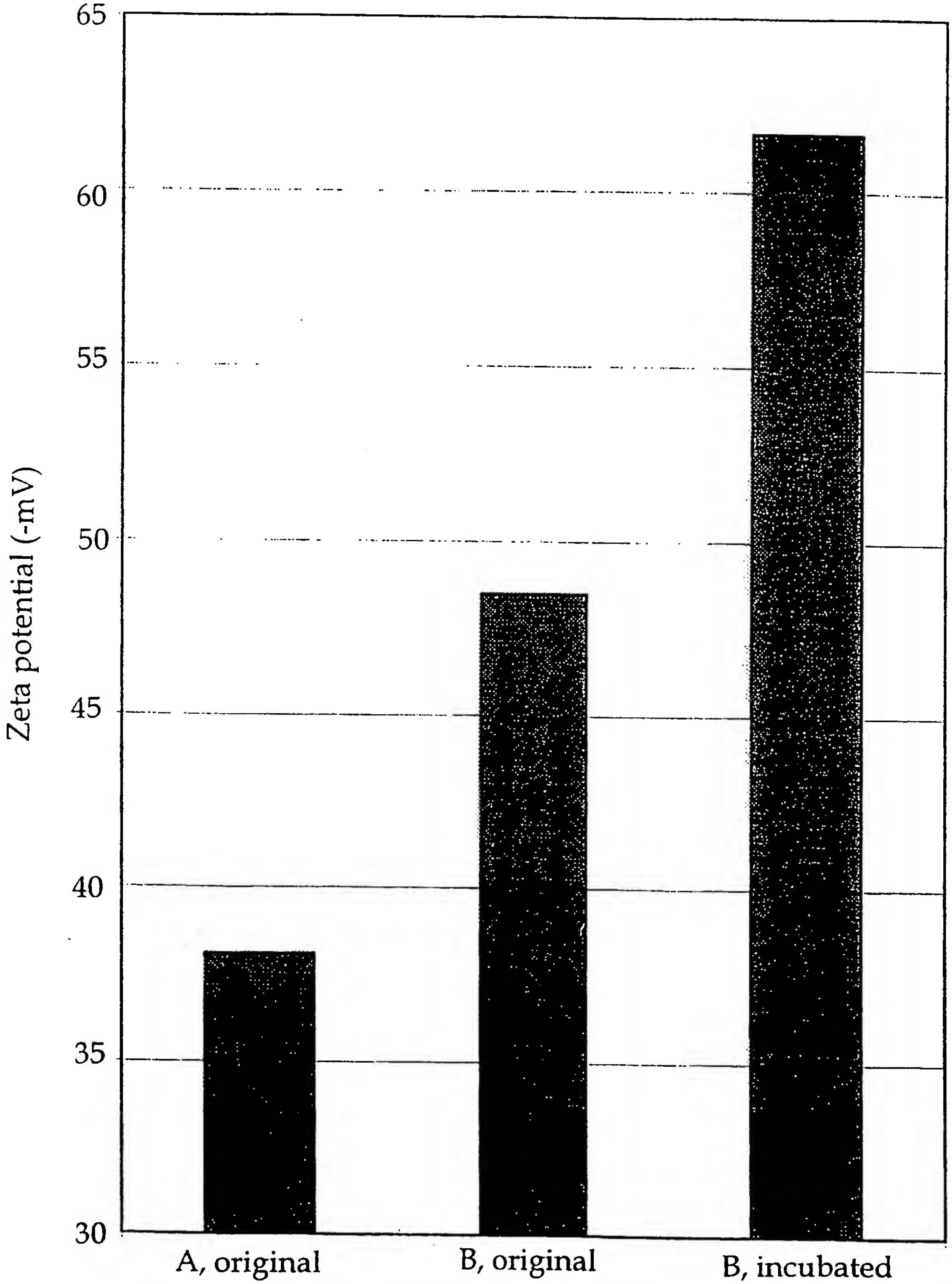

65
60
55
50
45
40
35
30
Zeta potential (-mV)
A, original
B, original
B, incubated

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/01407

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 9/16, A61K 9/127
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CLAIMS, USPATFULL, CAPLUS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Liposome technology/edited by Gregory Gregoriadis, Vol. 1, 2 rev.ed. 1992, "Liposome preparation using high-pressure homogenizers", page 49 - page 65	1-35
Y		38-40
	--	
X	US 5662932 A (SHIMON AMSELEM ET AL), 2 Sept 1997 (02.09.97)	1-35
Y		38-40
	--	
Y	EP 0298067 A1 (KABIVITRUM AB), 4 January 1989 (04.01.89)	38-40
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 1998	18.11.98

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Anneli Jönsson Telephone No. +46 8 782 25 00

International application No.
PCT/SE 98/01407

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patent Abstracts of Japan, abstract of JP 61-152632 A (dai ichi seiyaku co ltd), 11 July 1986 (11.07.86) --	27-37
A	EP 0663407 A1 (ROTKREUZSTIFTUNG ZENTRALLABORATORIUM), 19 July 1995 (19.07.95) --	1-40
A	US 5128318 A (DANIEL M. LEVINE ET AL), 7 July 1992 (07.07.92) --	1-40

INTERNATIONAL SEARCH REPORT
Information on patent family members

05/10/98

International application No.
PCT/SE 98/01407

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5662932 A	02/09/97	AU 676921 B	27/03/97
		AU 6916494 A	12/12/94
		AU 6948094 A	12/12/94
		CA 2162993 A	24/11/94
		EP 0702552 A	27/03/96
		IL 109641 D	00/00/00
		JP 8511245 T	26/11/96
		US 5576016 A	19/11/96
		US 5716637 A	10/02/98
		WO 9426252 A	24/11/94
		WO 9426255 A	24/11/94
EP 0298067 A1	04/01/89	SE 0298067 T3	
		AU 613379 B	01/08/91
		AU 1860788 A	05/01/89
		CA 1310871 A	01/12/92
		DE 3865756 A	28/11/91
		DK 167800 B	20/12/93
		DK 367988 A	03/01/89
		FI 92467 B,C	15/08/94
		FI 882981 A	03/01/89
		HR 920949 A	31/10/94
		JP 1080371 A	27/03/89
		JP 2108611 C	21/11/96
		JP 6061361 B	17/08/94
		MX 172551 B	03/01/93
		PT 87794 B	30/09/93
		SE 465065 B,C	22/07/91
		SE 8702735 A	03/01/89
		SI 8811284 A	30/06/97
		TR 25071 A	01/11/92
		US 4968299 A	06/11/90
		SE 464797 B,C	17/06/91
		SE 8801405 A	16/10/89
EP 0663407 A1	19/07/95	CA 2138925 A	01/07/95
		CN 1108662 A	20/09/95
		CZ 283620 B	13/05/98
		CZ 9403299 A	18/10/95
		FI 946199 A	01/07/95
		HU 70448 A	30/10/95
		HU 9403830 D	00/00/00
		JP 7242699 A	19/09/95
		NO 945101 A	03/07/95
		PL 306575 A	10/07/95
		US 5652339 A	29/07/97

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/10/98

International application No.

PCT/SE 98/01407

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5128318 A	07/07/92	AT 133423 T	15/02/96
		DE 3854940 D,T	11/07/96
		EP 0319557 A,B	14/06/89
		SE 0319557 T3	
		WO 8809345 A	01/12/88

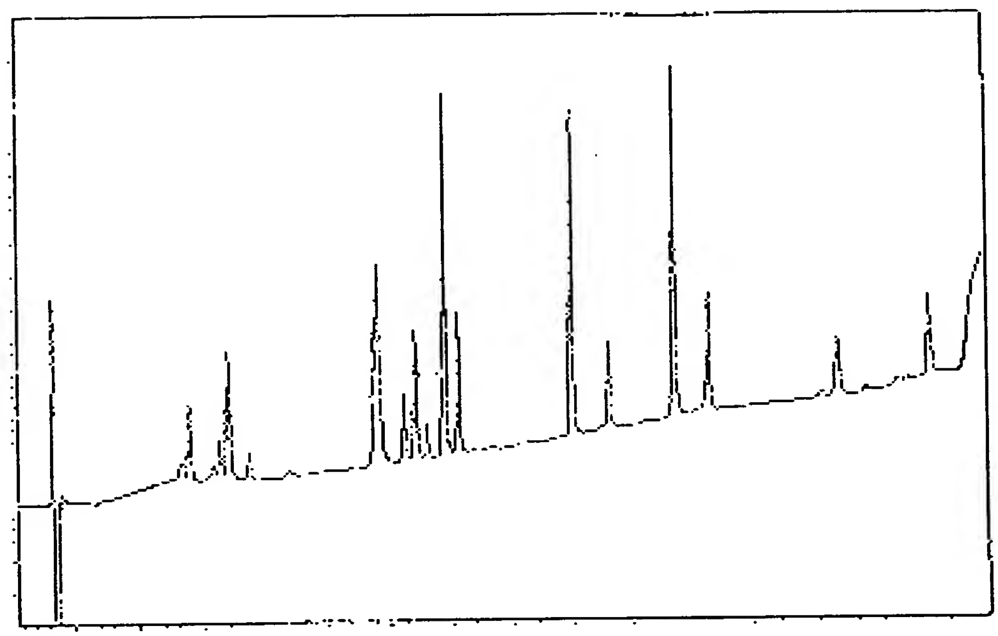


Fig. 1

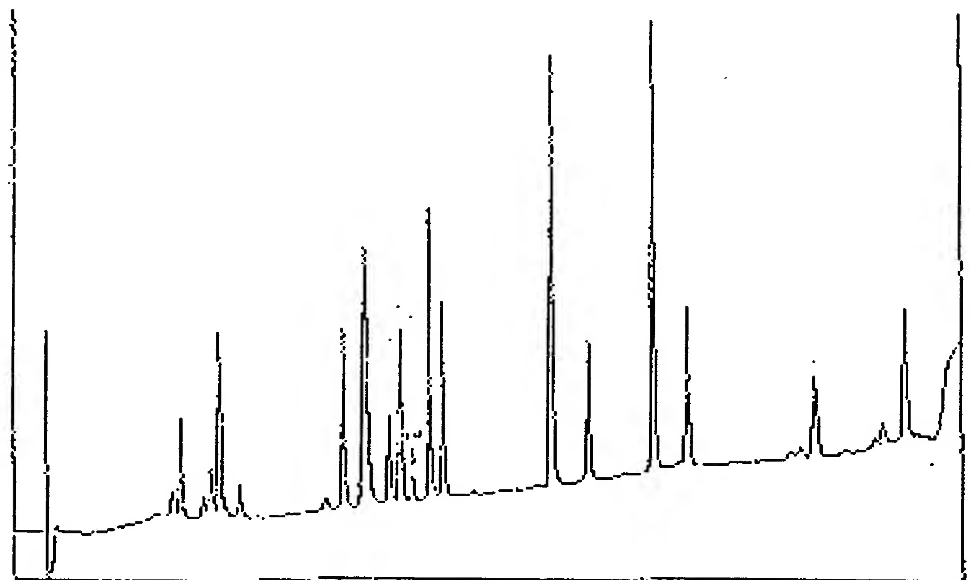


Fig. 2

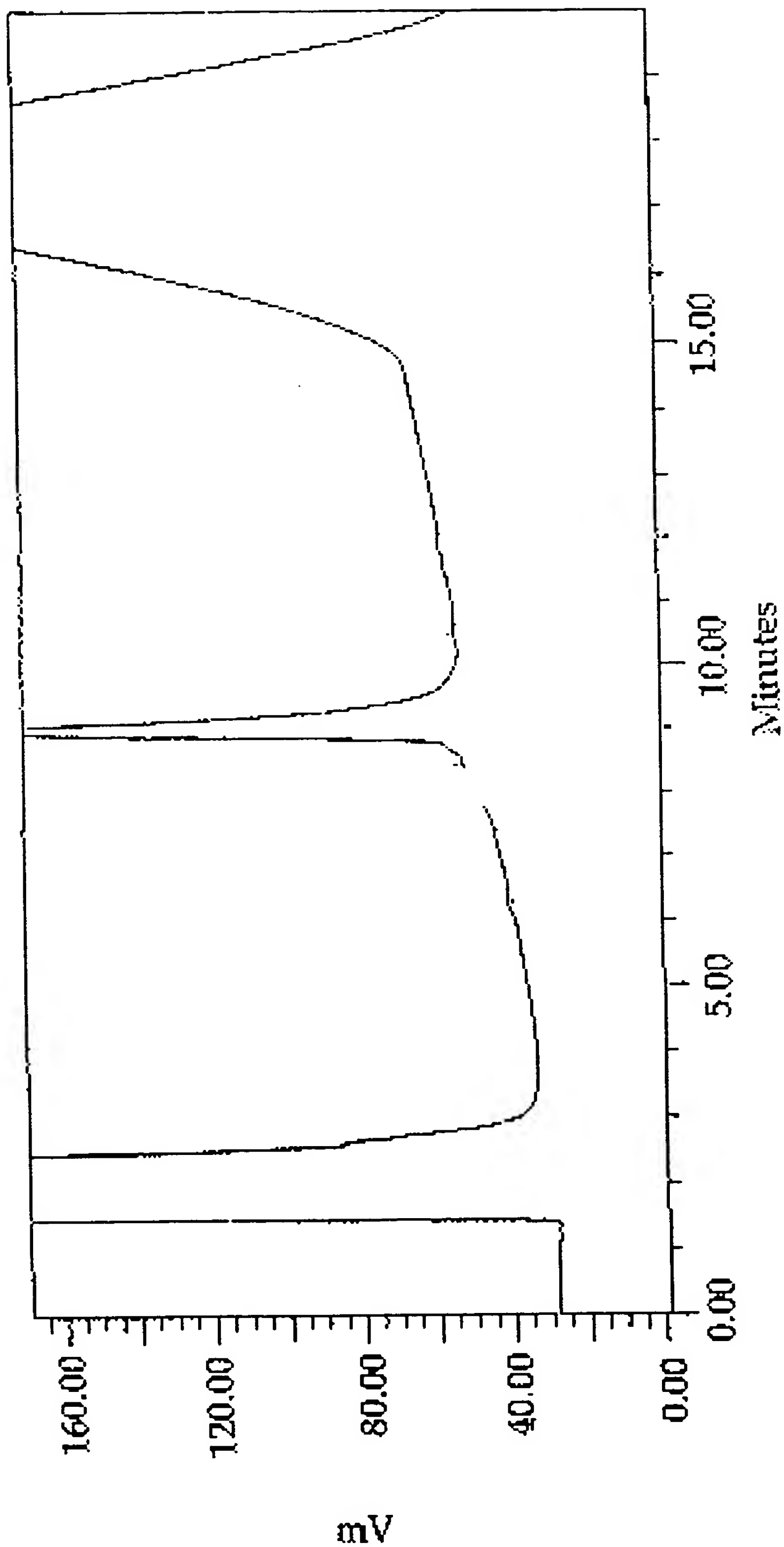


Fig. 3

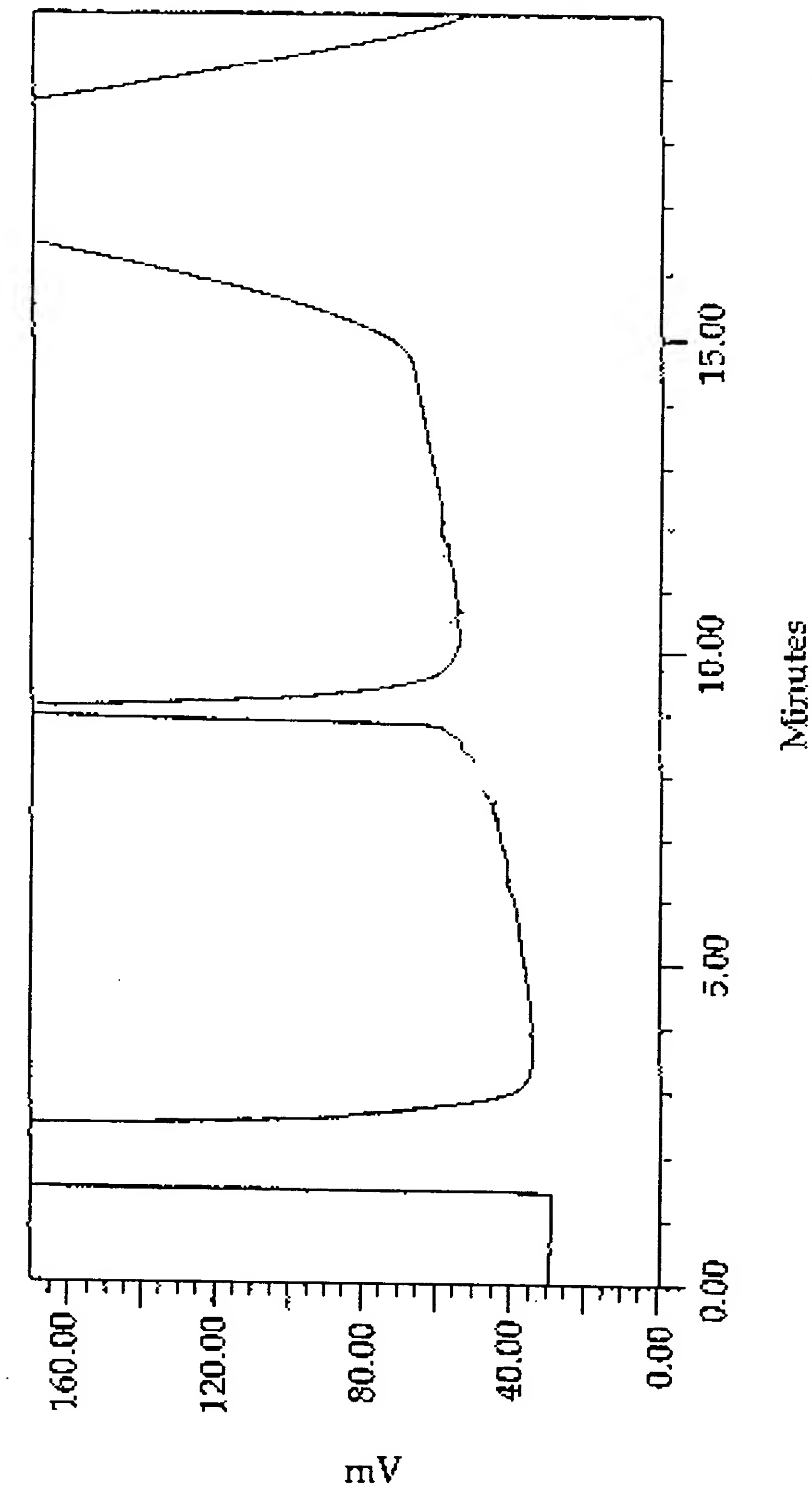


Fig. 4

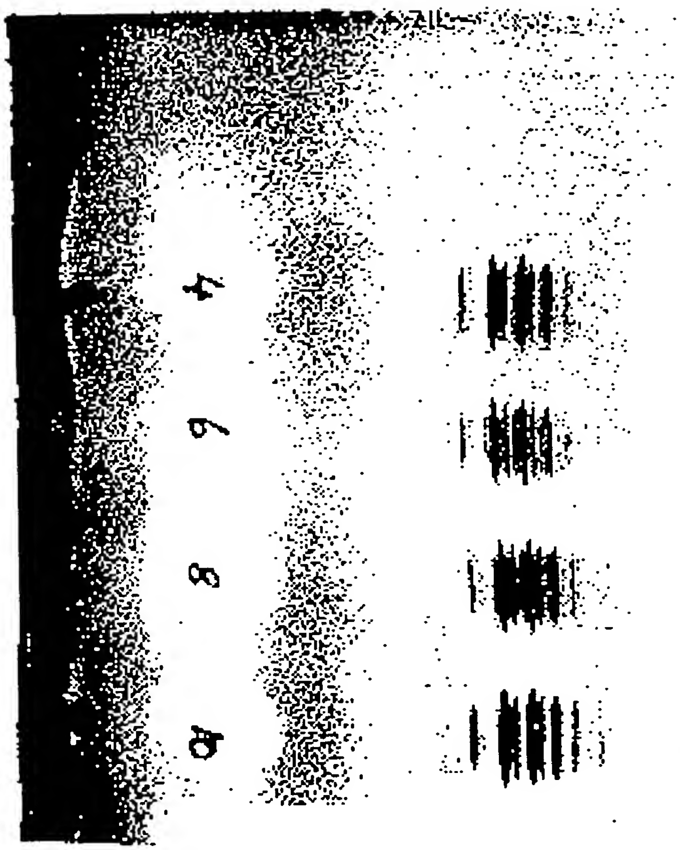


Fig. 5

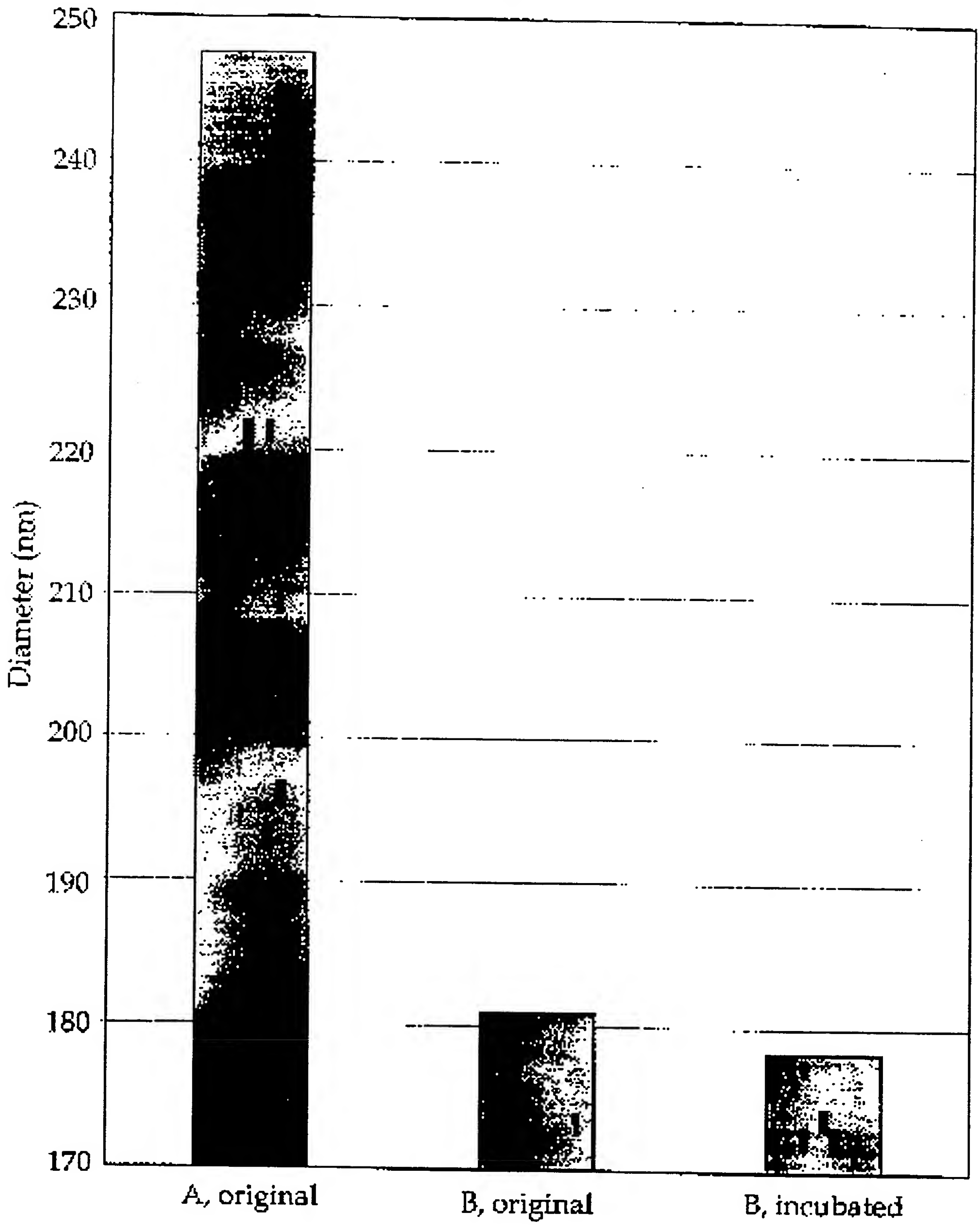

250
240
230
220
210
200
190
180
170
Diameter (nm)
A, original
B, original
B, incubated

Fig. 6

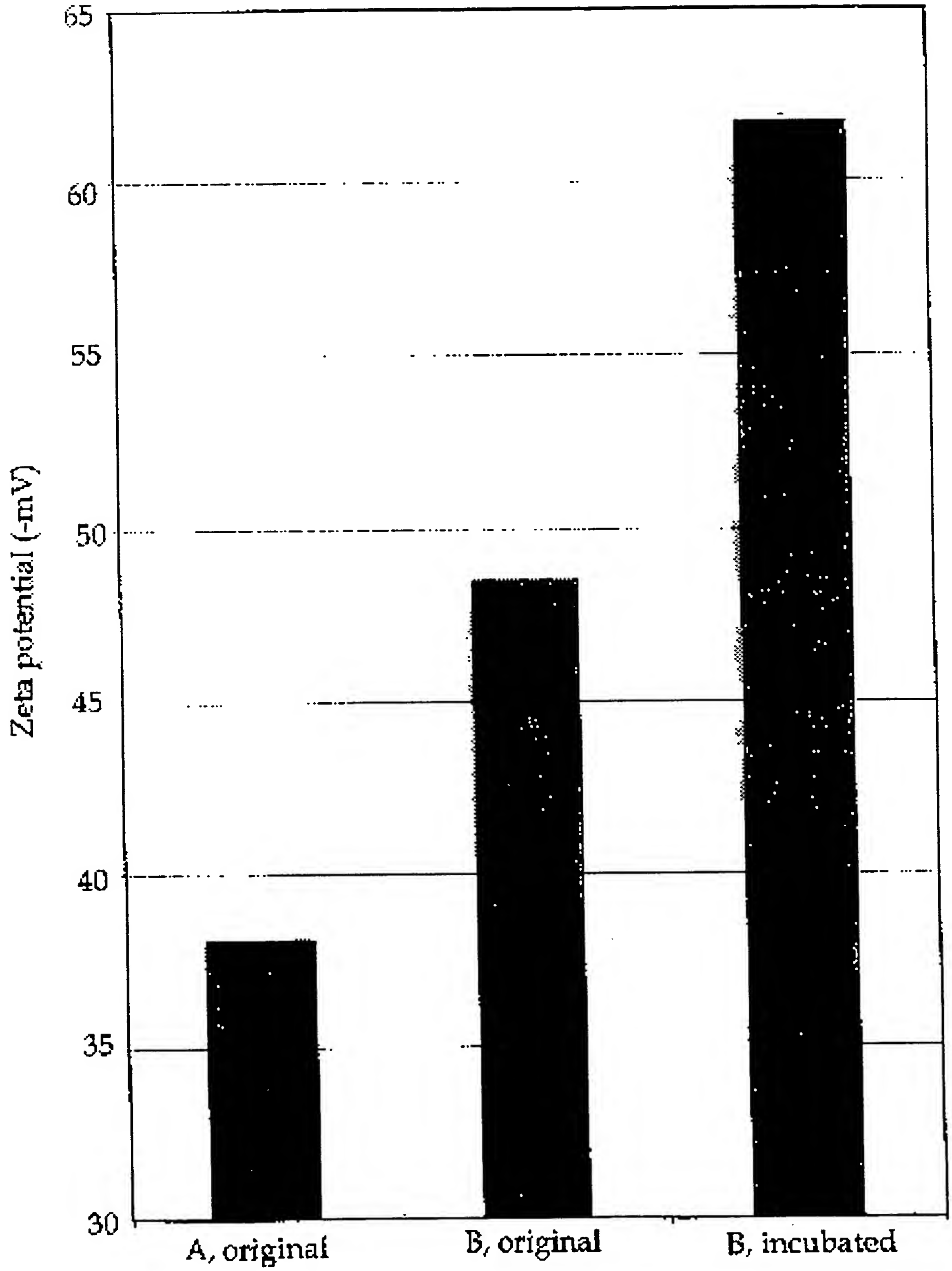

65
60
55
50
45
40
35
30
Zeta potential (-mV)
A, original
B, original
B, incubated

Fig. 7

C1
P.D. 14 Express Mail Label No. EV260609015US
p. 268-272 = (5)

XP-002117760

Degradability of crosslinked albumin as an arterial polyester prosthesis coating in *in vitro* and *in vivo* rat studies

Saïdi Benslimane, Robert Guidoin*, Paul-Emile Roy, Juan Friede, Jacques Hébert
Departments of Surgery and Pathology at Laval University, Biomaterials and Nuclear Medicine at St-François d'Assise Hospital and Immunology at CHUL, Québec, Canada

Dominique Domurado and Marie-Françoise Sigot-Luizard
Laboratoires de Technologie Enzymatique et de Biologie Cellulaire, Université de Compiègne, France

(Received 18 March 1985; accepted 15 September 1985)

In order to avoid the preclotting procedure in knitted polyester arterial prostheses and in woven models, compound polyester grafts have been proposed, containing preadsorbed collagen or albumin. Since we are currently investigating grafts impregnated with crosslinked albumin, it was decided to establish the degradation rate of this coating after stabilization with either glutaraldehyde (GA) or carbodiimide (CDI). Tests were performed *in vitro* by incubation in either PBS, plasma or pancreatin and *in vivo* by implantation in the abdominal cavity of rats. In PBS or plasma *in vitro*, the coatings were very stable (2% degradation after 144 h incubation), however, in pancreatin the CDI crosslinked albumin degraded much faster than the GA crosslinked albumin (more than 50% degradation in 12 h compared to less than 30% in 48 h). *In vivo* the degradation rates of the two types of crosslinked albumin were similar (almost all of the albumin having been lost after 4 weeks) but the cellular response was very different: a mild tissue reaction was observed with the CDI crosslinked coating whereas many foreign body giant cells were present on the GA crosslinked material.

Keywords: Prostheses, crosslinked albumin, arterial grafts, glutaraldehyde, carbodiimide, polyester, preclotting

The preclotting of arterial polyester prostheses is preferred by most surgeons for knitted structures[1] and it is recommended for woven structures[2], prior to implantation. The wall of the blood conduit must be made impervious by impregnation of its structure with a well-penetrating and anchored thrombotic matrix to prevent blood oozing. This operation is time-consuming. The quality of the resulting flow surface depends upon the graft structure and the blood properties of the patient. This manipulation prolongs the duration of the anaesthesia and increases the risks of bacteremic colonization[3]. The resulting flow surface can lead to structures likely to embolize[4]. These drawbacks can be limited in the compound prosthesis. Bascom[5], Humphries *et al.*[6] and Jordan *et al.*[7] developed collagen-coated grafts in the early sixties. Such a concept was reintroduced by Chvapil and Krajicek[8] and led to a commercial product (Hemashield, Meadox Medicals, Oakland, NJ, USA). Based on the antithrombogenecity and cytocompatibility of albumin coatings[9], polyester prostheses were impregnated with crosslinked albumin according to a protocol proposed by Gyurko *et al.*[10] and Domurado *et al.*[11,12]. Such a graft does not require any preclotting.

*Correspondence to: Dr Robert Guidoin, Laboratory of Experimental Surgery, Faculty of Medicine, Laval University, Québec, G1K 7P4, Canada.

Histologic observations made on glutaraldehyd (GA) crosslinked albuminated grafts implanted in the thoracic aorta of dogs for 1 month do not show any albumin remaining[13]. The focus of the present investigation was t quantify *in vitro* (in saline, plasma and pancreatin) and *in vivo* (in rats) the degradation rate of albumin crosslinked by means of either glutaraldehyde or carbodiimide.

MATERIALS AND METHODS

In vitro tests

Graft selection. We selected a woven graft (Woven de Bakey; Bard Implants Division, Billerica, MA, USA) and a texturized warp knitted graft (Vasculour II; Bard Implants). The grafts were cut into 1 cm lengths and coated according to the procedures which follow.

Albumin coating. We used two different crosslinking ag nts: glutaraldehyde (GA) (Merck, Darmstadt, FGR) and carbodiimide (CDI) [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride]; (Sigma, St Louis, MO, USA). The grafts were soaked in the following solution: 1.1 ml of bovine serum albumin (Sigma), containing 5 μCi of ^{125}I-albumin (Frost, Kirkland, PQ), 1 ml of phosphate buff red

saline (0.5 M, pH 7.5) and 0.15 ml (0.5 M, pH 7.5) of 25% GA. Th grafts were removed from the solution prior t compl te g lation and th cr sslinking r action was allowed to continue ov rnight at room temperatur . After th rough rinsing with distilled water, the residual aldehyd groups were neutralized with 0.13 M glycin in buff r and th grafts were freeze-dried. Th grafts wer ins rted into individual envelopes and gas sterilized in ethyl ne oxid using a standard hospital procedure.

For CDI crosslinking, grafts were immersed in 1.1 ml of 20% BSA/125 I-BSA with 1 ml of 0.2 M CDI in phosphate buffer (0.5 M, pH 4.75). The coated prostheses were then processed as described above. The amount of chemically attached albumin on each segment was evaluated, either by gravimetry or by radioactivity counting, (LKB Rack Gamma II,' LKB Bromma, Sweden) before washing.

Tests of degradation. The albuminated prostheses were inserted in sterile vials and then washed with constant stirring in a water bath shaker at 37°C. The incubating solutions were either phosphate buffered saline (PBS), bovine plasma, or pancreatin (0.1 g/l and 0.4 g/l), in solution with tris-(hydroxymethyl) aminomethane, HCl buffer at pH 7.6. Pancreatin (Sigma), was chosen because it contains a wide variety of hydrolytic enzymes including proteases and peptidases. Thus, the enzymes of pancreatin were capable of extensive hydrolytic action[14]. The experiments were done under sterile conditions to avoid any interference from microbial degradation.

Evaluation of the degradation rate. Individual coupons were withdrawn in triplicate from the solutions at 4, 24, 48, 72 and 144 h. After rinsing in distilled water, radioactivity counting was done for each segment in a LKB RACK Gamma II. Then the grafts were freeze-dried and weighed.

In vivo tests

Fabric selection. We selected a USCI Sauvage filamentous Dacron fabric (Bard Implants), which was cut into discs of 5 mm diam.

Albumin coating. We used the same procedure as for albuminating the prostheses except that the albumin was of rat origin (RSA: Sigma) containing 5 µCi of 125 I-RSA (NEN, Boston, Mass, USA).

Test of degradation. For the evaluation of the GA or CDI crosslinked albumin covered discs, 36 rats were selected and divided into 6 groups of 6 animals. The rats were anaesthetized with Halotane® (Hoechst Inc., Montreal, PQ, Canada) and prepared for surgery by shaving their abdominal regions and then preparing with betadine solution. Three discs were inserted into the peritoneal cavity with a 14 gauge needle and trocar.

Evaluation of the degradation. The discs were harvested at 1, 2, 7, 14, 21 and 28 d after implantation. The residual radioactivity was counted for each one.

Pathological analysis. The pathological stat of the explanted albuminated grafts w r studied by scanning electron microscopy acc rding to the stablished pr tocol and by light microscopy[15]. Briefly, th explants w re fixed in glutaraldehyde and dehydrated in a series of thanol dilutes (70, 90 and 100% ethanol). Then, the specimens w r embedded in histor sin (LKB), cut into 3 µm sections with an ultracut (Reich rt-Jung) and stained with methyl ne blu .

RESULTS

In vitro degradation

The results f washing the coated grafts in PBS showed that aft r a very small removal f albumin, th l vel stabilized. After 144 h of shaking at 37°C, the loss of albumin in wov n or velour prostheses measured by weighing and radioactivity counting was less than 2%.

In plasma, the two methods of evaluation (gravim try and radioactivity counting) gave contradictory results. Whereas we observed an increase in weight (5%) aft r plasma incubation, the radioactivity counting indicated a slight loss (2%). This might be due to the fact that plasma contains large amounts of proteins which can adsorb onto or diffuse into coated prostheses. In any case, there was no significant degradation of the albumin coating obtained by either GA or CDI crosslinking.

The removal of the chemically attached albumin in pancreatin solution (*Figure 1*) showed that the degradation rate of the coating depended upon the crosslinking agent. The GA crosslinked albumin degradation is linear with tim and depends upon pancreatin concentration. With the 0.1 g/l pancreatin solution, the loss of the coating was less than 30% at up to 144 h. This degradation was increased with higher pancreatin concentration. With 0.4 g/l, it was about 70% after 144 h of incubation. With CDI, th degradation was very rapid; at 24 h, the release of albumin coating was already 80%. It degraded totally at 144 h. We observed approximately the same rate of degradation either with 0.1 g/l or 0.4 g/l of pancreatin solution (*Figure 1a, b*).

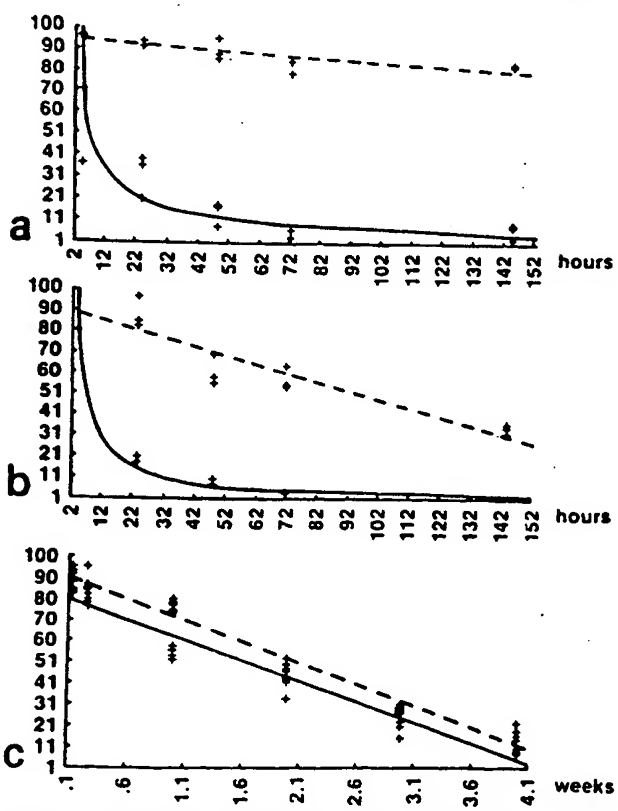


Figure 1 Percentage of crosslinked albumin still present in GA - - -, and CDI——, at various time intervals after in vitro exposure to pancreatin [(a), 0.1 g/l; (b), 0.4 g/l] or in vivo implantation in the peritoneal cavity of rats (c).

In vivo degradation

Degradation rate of albumin. Th results of the *in vivo* experiments depicted in *Figure 1* show that:

(i) Th two coatings are degradabl *in vivo*;
(ii) The removal of the albumin coating is rapid in the first 24 h, and then becomes lin ar with time;
(iii) All the attached albumin is rel ased at 5 wk;
(iv) The rate of degradation is approximately the same with either GA or CDI crosslinking.

Tissue reactions. The cellular reactions were rapid. At 24 h, the grafts were invaded by phagocytic cells. This cellular infiltration was seen even between the polyester yarns (*Figure 2 a, b*). Nevertheless, the CDI crosslinked grafts seemed more encapsulated by a dense, regular network of fibrillar material (*Figure 4 a, b*). After 1 wk, the fibrillar matrix density decreased and the granulocytic cells were found to be in contact with the albuminated polyester. At 4 wk, the GA crosslinked grafts were still infiltrated by a dense granulocytic tissue, with many giant cells (*Figures 3 a* and *5 a*). The tissue reactions with CDI crosslinked grafts were mild at that time and only rarely were granulocytes observed (*Figures 3 b* and *5 b*).

DISCUSSION

Radioactivity counting is more sensitive and reproducible for albumin assay than the gravimetric method, because it is specific for radi labelled albumin and does not include proteins adsorbed from the incubant in the assay.

The crosslinking mechanisms of glutaraldehyd and carbodiimid are different. Wher as GA crosslinks amino groups on the sam or different protein molecules by means of a chain made of several of its own molecul s[16]. CDI activat s a carboxyl group to ultimately form a p ptidic bond with an amin gr up of the same protein (if st ric hindrance permits) or of another protein[17].

Since only peptidic bonds are present in coatings made by use of CDI, it is understandable that a protease mixture such as pancreatin is efficient in solubilizing it. In th case of GA, it is not known whether enzymes ar abl to break the bonds formed, or if it is necessary to hydrolyse the proteins to release the untransformed chemical crosslink.

Since GA uses a chain of variable length to crosslink amino groups, it can more easily form intra- and int r-molecular bonds. If the crosslink density is higher with GA than with CDI the proteases have more difficulty diffusing into the albumin mesh and the hydrolysis takes a long r time.

In vivo, the kinetics of the degradation of CDI crosslinked albumin is completely different to its *in vitro* counterpart and is similar to the degradation of th GA coating. On the one hand, *in vivo* proteolysis is effective, since GA albumin disappears at a rate approximately equivalent to the rate obtained *in vitro* with 0.2 g/l f pancreatin, yet on the other hand it is limited, as the degradation rate of CDI albumin is considerably reduced compared to that *in vitro.* This phenomenon is likely to be du

Figure 2 *Histologic cross-section of the tissue response of the samples implanted for 24 h in the peritoneal cavity of rats. The methylene blue stainings illustrate the market nuclear cell reaction either with GA (a) or CDI (b) crosslinked albumin. (× 448).*

Figure 3 *Histologic cross-section of the tissue response of the sample implanted for 28 d in the peritoneal cavity of rats. The methylene blue stainings illustrate a marked nuclear and giant cell reaction associated with the GA crosslinked albumin (a) compared to a mild cellular reaction with the CDI crosslinked albumin (b). (× 448).*

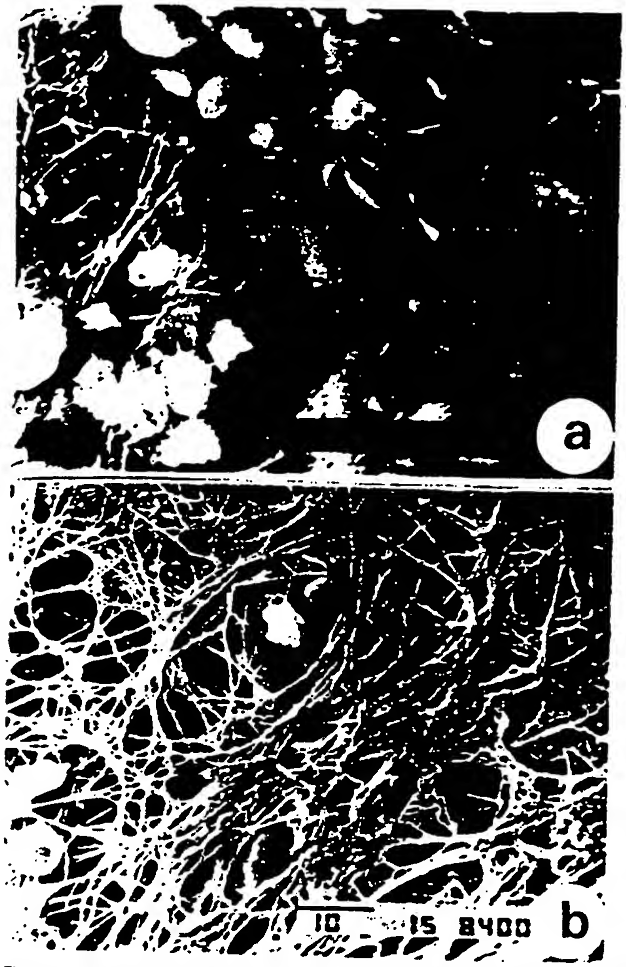


Figure 4 *SEMs of the surface of the specimens 24 h after implantation showing a marked difference in the tissue response: the polyester coated with GA crosslinked albumin has a very small fibrillar network (a) compared to that of CDI crosslinked albumin (b).*

Figure 5 *SEMs of the surface of the specimens 28 d after inplantation. The tissue reaction caused by the GA crosslinked albumin is still very strong (a) whereas inflammatory cells are no longer visible on the CDI crosslinked albumin (b).*

to the differences between degradation mechanisms, namely, pancreatin *in vitro* and phagocytic cells *in vivo*. The pathological analysis showed two different patterns of cellular reaction depending upon the crosslinking agent used. Grafts coated with CDI albumin contained mainly neutrophilic cells (acute inflammation type) and the tissue response was very mild 4 weeks after implantation. The ease of proteolysis or the presence of only amino acid after degradation might be responsible for this. In contrast, the tissue response observed in GA albumin grafts involved macrophages and foreign body giant cells (chronic inflammation type) and was still very active 4 weeks after implantation. The difficulty of proteolysis and the specific action of GA-derived crosslinks are the two factors suspected to account for this difference.

CONCLUSION

The present work demonstrates the stability of the albumin crosslinking ither by GA or CDI in PBS and plasma, and it biodegradability by enzymatic hydrolysis. The *in vivo* behaviour of CDI crosslinking compared to that of GA crosslinking combined with th abs nc f residual ch micals aft r hydrolysis and of a chronic inflammatory reaction speak for the use of th form r for therapeutical applications.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada (grant MA 6764). The authors are indebted to J. Bastien, S. Bourassa, H. Desbiens, D. Gagnon, D. Martel, L. Martin, R. Paynter and C. Mongrain for valuable discussions and technical assistance. The gift of prostheses and fabrics by Bard Implants Division is gratefully acknowledged.

REFERENCES

1 Yates, S.G., Barros d'Sa, A.A.B., Berger, K., Fernandez, L.G., Wood, S.J., Rittenhouse, E.A., Davis, C.C., Mansfield, P.B. and Sauvage, S.L., The preclotting of porous arterial prostheses, *Ann. Surg.* 1978, **188**, 611-622

2 Topping, C., Guidoin, R., Roy, J., Picard, R., Bernier, J., Gosselin, C., Blais, P., Martin, L., Marois, M. and Noël, H.P., La précoagulation des prothèses vasculaires en Dacron tissé, *J. Chir.* 1980, **117**, 403-409

3 Wooster, D.L., Krajden, S. and Louch, R.E., Intraoperative bacterial contamination of vascular grafts, *Roy. Coll. Phys. Surg. Can. 53rd Annual meeting*, Montreal, Sept., 1984

4 Gloviczki, P., Hollier, L.H., Hoffman, E.A., Vlabe, G., Trasbek, V.F. and Kaye, M.P., The effect of preclotting on surface thrombogenicity and thromboembolic complications of Dacron grafts in the canine thoracic aorta, *J. Thorac. Cardiovasc. Surg.* 1984, **88**, 253-258

5 Bascom, J.V., Gelatin sealing to prevent blood loss from knitted arterial grafts, *Surgery* 1961, **50**, 504-512

6 Humphries, A.W., Hawk, W.A. and Cuthbertson, A.M., Arterial prosthesis of collagen impregnated Dacron tulle, *Surgery* 1961, **50**, 947-954

7 Jordan, G.L., Stump, M.M., Allen, J., DeBakey, M.E. and Halpert, B., Gelatin impregnated Dacron prosthesis implanted in porcine thoracic aorta, *Surgery* 1963, **53**, 45–49

8 Chvapil, M. and Krajicek, M., Use of collagen in the construction of an arterial prosthesis, *Med. Trends Vasc. Surg.* 1970, **1**, 120–140

9 Packman, M.A., Evans, G., Glynn, M.F. and Mustard, J.F., The effect of plasma proteins on the interaction of platelets with glass surface, *J. Lab. Clin. Med.* 1969, **73**, 686–697

10 Gyurko, G.Y., Szücs, J., Banhegyi, J., Kenyeres, I., Szikszai, P., Noviczki, M. and Posze, G., Experimental experience with polyester velour in vascular plastic surgery, *Acta Chir. Acad. Sc. Hung.* 1974, **15**, 35–43

11 Domurado, D., Thomas, D. and Broun, G., A new method for producing proteic coatings, *J. Biomed. Mater. Res.* 1975, **9**, 109–110

12 Domurado, D., Guidoin, R., Marois, M., Martin, L., Gosselin, C. and Awad, J., Albuminated Dacron prostheses as improved blood vessel substitutes, *J. Bioeng.* 1978, **2**, 79–91

13 Guidoin, R., Snyder, R., Martin, L., Botzko, K., Marois, M., Awad, J., King, M., Domurado, D., Bedros, M. and Gosselin, C., Albumin coating of a knitted polyester arterial prosthesis: an alternative to preclotting, *Ann. Thorac. Surg.* 1984, **37**, 457–465

14 Needham, D.K., Buchanan, D.R., Gupta, B.S. and Horton, H.R., Enzymatic removal of formalin-fixed tissues from polyester arteries and its effect on physical properties of fibers, *J. Biomed. Mater. Res.* 1982, **16**, 659–668

15 Guidoin, R., Gosselin, C., Martin, L., Marois, M., Laroche, F., King, M., Gunasekera, K., Domurado, D., Sigot-Luizard, M. -F. and Blais, P., Polyester prostheses as substitutes in the thoracic aorta of dogs. I. Evaluation of commercial prostheses, *J. Biomed. Mater. Res.* 1983, **17**, 1049–1077

16 Monsan, P., Puzo, G. and Mazarguil, H., Etude du mécanisme d'éstablissement des liaisons glutaraldéhyde-protéines, *Biochimie* 1975, **57**, 1281–1292

17 Sheehan, J.C. and Hlavka, J.J., The cross-linking of gelatin using a water soluble carbodiimide, *J. Am. Chem. Soc.* 1957, **79**, 4528–4529